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THE ASSOCIATION OF INFLAMMATORY BIOMARKERS WITH CARDIOVASCULAR EVENTS: A LONG AND WINDING PATH

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The Association of Inflammatory Biomarkers with Cardiovascular Events: a Long and Winding Path
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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A Mamá y Papá

"Learning is a treasure that will follow its owner everywhere"

"When the winds of change blow, some people build walls and others build windmills"

Chinese proverbs

ABSTRACT

Aims: The overall aim of this thesis was to investigate the association of inflammatory biomarkers with cardiovascular disease (CVD) with the final scope to identify potential novel predictors of incident cardiovascular events (CVE). The specific objectives were: to study the association between levels of IL8 and the genetic variants at the *IL8* and *IL8* receptor genes with the occurrence of myocardial infarction (MI) and to explore if these genetic variants regulate IL8 levels (Study 1); to identify novel genetic variants associated with IL8 levels (Study II); to study the association of IL8 levels with the risk of CVE (Study III) and, finally, to investigate the association between the levels of soluble IL6 receptors (sIL6R and sgp130) with the occurrence of MI (Study IV).

Materials and Methods: Study I, II and IV were based on the Stockholm Heart Epidemiology Program (SHEEP), a population-based case-control study of incident MI performed between 1992 and 1994 at the ten emergency hospitals within the county of Stockholm. Controls were randomly selected from the study base, using density sampling and matching for age, sex and hospital catchment area. The analyses performed in this thesis were restricted to non-fatal cases (n=1213) and matched controls (n=1561), with available biomarkers data. Exposure information was available from questionnaires, anthropometric measurements, blood samples and medical records. Study III was based on a cohort of 60-year-old men and women from Stockholm (60YO), a population-based prospective study performed between 1997 and 1999 in Stockholm County. A total of 4232 individuals (78%) agreed to be enrolled in the study. Participants were followed for an average of 14.5 years for the assessment of CVE and cardiovascular mortality. Up to December 31, 2012, n= 491 cases were recorded. A nested-case control was conducted based on the full enumerated cohort. Controls (n= 981) were randomly selected from the study base having the same sex as the case, being alive, and not been classified as a case at the date of the CVE (+/- 60 days).

Results and Conclusions: Circulating levels of IL8 were associated with a reduced occurrence of non-fatal MI in the SHEEP population aged 45-70 years (Study I), whereas no association was observed with the risk of CVE- defined as fatal and non-fatal MI, fatal and non-fatal stroke and hospitalization due to angina pectoris in the 60YO cohort (Study III). Median levels of IL8 did not vary according to *IL8* and *IL8* receptor genetic variants (Study I). A SNP rs12075 A/G, Asp42Gly, mapping at the Duffy antigen receptor for chemokines (*DARC*) gene on chromosome 1 was associated with circulating IL8 in serum, but not in plasma. Homozygous carriers of the G allele had lower levels of IL8 (Study II). These findings were replicated in independent populations. SIL6R and sgp130 had opposing associations with MI. Sgp130 was an effect modifier of the association of sIL6R with MI and there was an indication of a possible interaction between high sIL6R and low sgp130 with the risk of MI (Study IV).

Our results suggest that differences in baseline characteristics of a study population, genetic predisposition and genetic regulation of biomarkers should be taken into account when interpreting the results of associations of biomarkers with CVD risk. In a broader sense, the role of molecular pathways should be evaluated in studies aimed at analysing the risk associated with complex outcomes rather than applying single biomarker approaches.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following original articles/manuscript, which will be referred to in the text by their Roman numerals

I. **Velásquez IM**, Frumento P, Johansson K, Berglund A, de Faire U, Leander K, Gigante B.
Association of interleukin 8 with myocardial infarction: results from the Stockholm Heart Epidemiology Program.
Int J Cardiol. 2014 Mar 1;172(1):173-8

II. **Moreno Velásquez I**, Kumar J, Björkbacka H, Nilsson J, Silveira A, Leander K, Berglund A, Strawbridge RJ, Ärnlöv J, Melander O, Almgren P, Lind L, Hamsten A, de Faire U, Gigante B.
Duffy antigen receptor genetic variant and the association with Interleukin 8 levels.
Cytokine. 2015 Jan 31;72(2):178-184.

III. **Moreno Velásquez I**, Leander K, Berglund A, Gajulapuri A, de Faire U, Gigante B.
Association of serum Interleukin 8 levels with incident cardiovascular events. Results from the cohort of 60 years old men and women from Stockholm using a nested case-control design.
Submitted.

IV. **Moreno Velásquez I*** , Golabkesh Z*, Källberg H, Leander K, de Faire U, Gigante B.
Circulating levels of Interleukin 6 soluble receptor and its natural antagonist, sgp130, and the risk of myocardial infarction.
Atherosclerosis. 2015Apr 14; 240(2):477-481.

**Contributed equally*

RELATED PUBLICATIONS

V. **Moreno Velásquez I**, Ärnlöv J, Leander K, Lind L, Gigante B, Carlsson AC. Interleukin-8 is associated with increased total mortality in women but not in men-findings from a community-based cohort of elderly.
Ann Med. 2015 Feb;47(1):28-33

VI. Gigante B, Strawbridge RJ*, **Velásquez IM***, Golabkesh Z, Silveira A, Goel A, Baldassarre D, Veglia F, Tremoli E, Clarke R, Watkins H, Hamsten A, Humphries SE, de Faire U.
Analysis of the role of interleukin 6 receptor haplotypes in the regulation of circulating levels of inflammatory biomarkers and risk of coronary heart disease.
PLos One. 2015 Mar 17;10(3)

**Contributed equally*

CONTENTS

LIST OF ABBREVIATIONS

ACS	Acute coronary syndrome
AP	Attributable proportion
ASA	Acetyl salicylic acid
BMI	Body mass index
CABGs	Coronary artery bypass graft surgery
CHD	Coronary heart disease
CHF	Chronic heart failure
Chr	Chromosome
CI	Confidence interval
CoV	Coefficient of variation
CRP	C reactive protein
CV	Cardiovascular
CVD	Cardiovascular disease
DALYs	Disability adjusted life years
ECG	Electrocardiogram
ECs	Endothelial cells
ESS	Endothelial shear stress
GWAs	Genome wide association studies
HRT	Hormone replacement therapy
HUVEC	Human umbilical vein endothelial cells
IL6	Interleukin 6
IL8	Interleukin 8
IL8R	Interleukin 8 receptors
IQR	Interquartile range
IRR	Incidence rate ratio
LDL	Low density-lipoprotein
MAF	Minor allele frequency
MI	Myocardial infarction
OR	Odds ratio

PC	Principal components
PCI	Percutaneous coronary intervention
PMN	Polymorphonuclear
S	Synergy index
SCD	Sudden cardiac death
SNP	Single nucleotide polymorphism
sgp130	Soluble glycoprotein 130
sIL6R	Soluble Interleukin 6 receptor
STEMI	ST segment elevation myocardial infarction
WHO	World Health Organization

1 INTRODUCTION

Cardiovascular (CV) disease (CVD) is a global public health concern. Despite advances in diagnosis and treatment, by 2020, CVD will become the leading cause of death and disability worldwide, with the numbers of fatalities expected to increase to more than 24 million by 2030 (1). Modifiable (e.g., smoking, physical inactivity, hypertension, diabetes, hyper-dyslipidaemias, obesity) and non-modifiable conditions (family history, age, and sex) constitute risk factors for CVD (2).

Coronary heart disease (CHD), the most common manifestation of CVD, accounted for 77.7 million disability adjusted life years (DALYs) in the adult population in 2010, and the burden of DALYs was projected to increase by 35% from 2004 to 2030 (3, 4). A pivotal study, the Framingham Heart cohort, first of its kind, studied multiple risk factors that determine a person´s risk for CHD (5, 6). Today, much of the decline in CVD burden is due to reduction in risk factors which this study contributed to identify (7).

However, current algorithms that are commonly used, such as the Framingham risk score and lipid parameters fail to identify all individuals at risk of a CV event (CVE): one out of five patients who suffer from CHD lacks one or more of the four conventional risk factors, which include hyperlipidaemia, diabetes mellitus, smoking and hypertension (8). This suggests that the participation of other additional factors contribute to the occurrence of a CVE. Moreover, individuals with one or only a few modifiable risk factors are the least likely to be targeted for preventive therapies (9, 10).

In terms of cost-effectiveness, identifying novel factors that target the segment of the population at risk of CVD whom are not identified by the commonly used algorithms may have substantial public health implications. Therefore, in recent years, identification of novel biomarkers able to predict CV risk above and beyond the known CV risk factors has been an emerging field in epidemiological studies (9).

Inflammation plays a role in the development of CHD (11). Thus, this thesis is focused on the association between inflammatory biomarkers and occurrence of CVE. Three biomarkers *Interleukin 8, soluble Interleukin 6 receptor and soluble glycoprotein 30* have been evaluated as exposure.

2 BACKGROUND

2.1 DEFINITIONS

CVD is a broad term which defines a group of disorders of the heart and blood vessels including CHD, cerebrovascular diseases, peripheral arterial disease, congenital heart disease, rheumatic heart disease, deep vein thrombosis and pulmonary embolisms (12).

CHD refers to all the spectrum of clinical manifestation of atherosclerosis in the coronary arteries from stable angina to acute coronary syndromes (ACS) (i.e., unstable angina and myocardial infarction (MI)).

In the present thesis, the following diseases were considered as outcomes:

Non-fatal MI: (Study I, II and IV) and CVE: a combined endpoint of CHD and ischemic stroke, either fatal or non-fatal (Study III).

2.2 BURDEN OF DISEASE

Population ageing is driving the global epidemic of chronic diseases (3). Despite the decline in mortality rate attributable to CVD in Western Europe and North America nowadays (13, 14), CVD is still the leading cause of death worldwide. Eighty percent of all CVD deaths occur disproportionately in low-and middle-income countries (15).

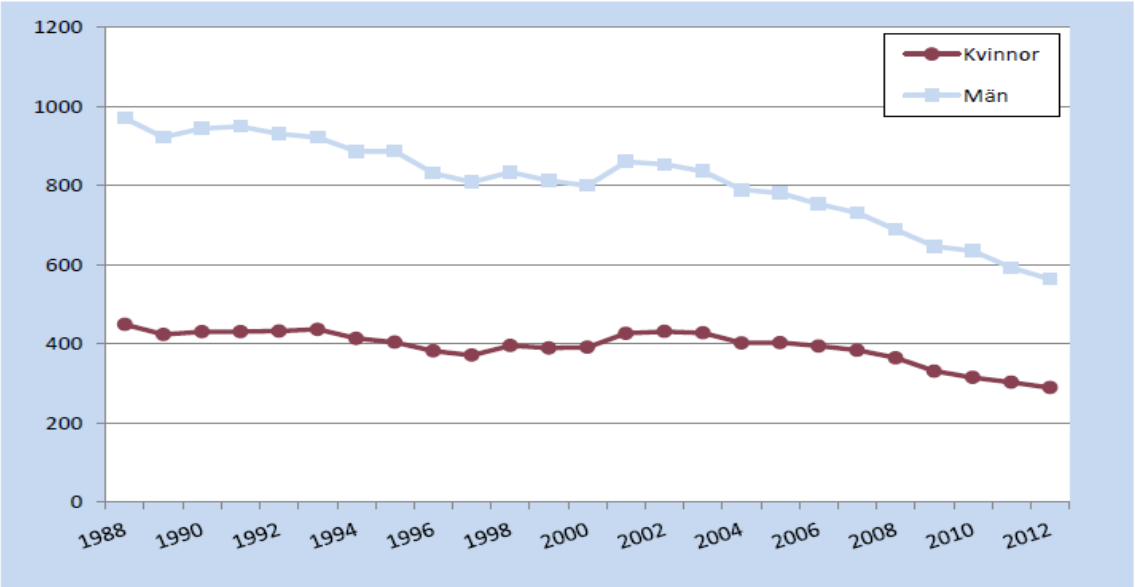


Figure 1. *Age standardized incidence of acute myocardial infarction per 100000 inhabitants 20 years old and older according to sex and calendar year, 1998-2012. Åldersstandardiserad Socialstyrensen MI trends. Hjärtinfarkter 1988–2012.*

In Sweden, during the period 1988-2000, the age standardized incidence of acute MI decreased overall (*Figure 1*). In the year 2001, the introduction of the novel diagnostic criteria for MI *(see section 2.3.1.2)* increased the age-standardized incidence, and by the year 2004, the incidence fell to the same levels as before the introduction of the new criteria, and then decreased each year after that. The MI mortality rate and case fatality has decreased in both, men and women. On the other hand, a recent study has shown that the overall incidences of MI and ischemic stroke decreased over time among men, but were stable over time among women in Sweden (16).

2.3 GENERAL OVERVIEW OF CHD

2.3.1.1 Pathophysiology of MI

MI occurs as a result of rupture or superficial erosion of an atherosclerotic plaque. This event triggers the development of an occlusive thrombus in the coronary artery causing deprivation of oxygen supply and acute ischemia in the tissue distal to the occluded artery.

Within the last few decades, overflowing evidence has characterized atherosclerosis as a chronic, inflammatory, fibroproliferative process with immune components (17, 18). However, the complexity of this process and the mechanisms underlying inflammation are only partly understood. The process of atherogenesis is latent for years before it becomes clinically significant and yet, not all atherosclerotic plaques lead to a clinically overt disease (19).

Although the entire vasculature is exposed to the atherogenic effects of the systemic risk factors, atherosclerotic lesions form at specific regions of the arterial tree, thus the local hemodynamic forces, e.g. low endothelial shear stress (EES), are implicated in atherogenesis (20).

The chain of events that precede the rupture of the atherosclerotic plaque can be summarized as reported in *Figure 2 (21).*

1. Vascular cells are exposed to excess low-density lipoprotein (LDL) causing endothelial activation/dysfunction and the internalization and deposition of lipids in sub-intimal space.
2. Recruitment of inflammatory cells, (neutrophiles, monocytes), one of the crucial events in atherogenesis, is triggered.

3. Once resident in the artery wall, monocytes undergo structural and functional alterations via macrophage colony-stimulating factor, and differentiate into tissue macrophages.
4. Herein, macrophages sustain an inflammatory reaction and increase the oxidative stress. With the uptake of oxidized LDL (oxLDL) into the atherosclerotic lesions they form foam cells.
5. Smooth muscle cells (SMCs) from the tunica media -the middle layer of the artery wall- may emigrate into the subintimal space to participate in the atheroma formation or proliferate and produce collagen and elastin to form a fibrous cap that covers the plaque.

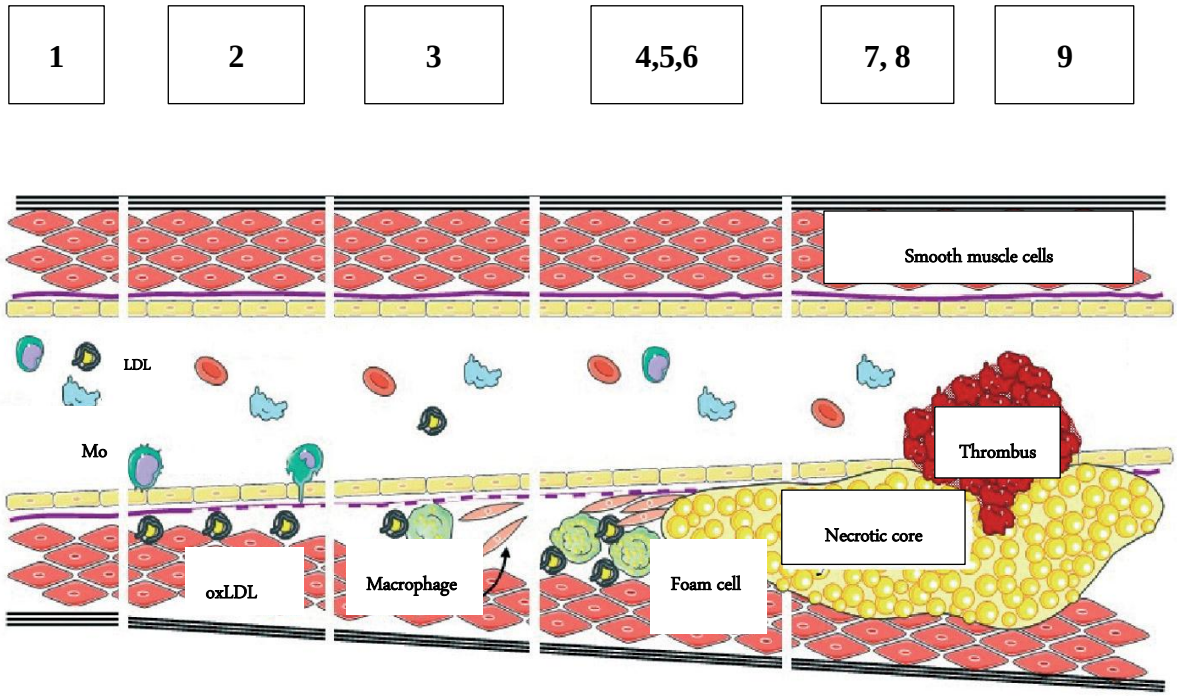


Figure 2. *Initiation and progress of atherosclerosis in the arterial wall. Adapted from Rodriguez JA et al., 2007. Metalloproteases, Vascular Remodeling, and Atherothrombosis Syndromes (21). Mo: monocytes*

6. The content of the plaque is then a collection of foam cells, some of which die with the consequent release of lipids that accumulate extracellularly. If the clearance of dead cells is inefficient, accumulation of cellular debris and extracellular lipids form a lipid rich pool known as the necrotic core of the plaque (22).
7. The necrotic core expands and the fibrous cap covering the plaque can be broken down by matrix degrading metalloproteinases (MMPs) secreted by macrophages. As

a consequence, the plaque becomes thinner and erodes leading to the so called-*unstable or vulnerable plaque.*

8. The disruption of the cap leads to the burst of the plaque and exposes the necrotic core to clotting factors and platelets in the circulation.
9. Thereafter, the interaction between exposed atherosclerotic plaque components, blood cells and coagulation factors leads eventually to platelet activation and aggregation. Thrombus is formed at the endothelial surface in the lumen of the arteries leading to a partial or total occlusion of the vessel, with consequent cell death of the myocardium distal to the occlusion of which ACS is the clinical manifestation (23).

2.3.1.2 **Evolution of MI diagnostic criteria**

The definition of MI has evolved over time as more sensitive and specific biomarkers have been developed. By the end of 1970´s, the World Health Organization (WHO) consensus for the definition of MI was based on a combination of two of the following three characteristics: typical symptoms (e.g., chest discomfort), enzyme rise and a typical electrocardiogram (ECG) pattern involving the development of Q waves (24).

Later on, the diagnostic criteria for MI was redefined by the European Society of Cardiology and the American College of Cardiology in 2000 (25). This first universal MI definition emphasized the use of myocardial tissue specific biomarkers (troponin) to reflect the myocardial necrosis that may occur without symptoms and in the absence of ECG changes. Any necrosis in the setting of myocardial ischaemia was labelled as MI. The implementation of these criteria, as shown in several epidemiological studies, leads to a significant rise in the rate of diagnosis of acute MI (26, 27), as outlined earlier (section 2.2). Shortly after, the Global Task Force, made up of the European Society of Cardiology, American College of Cardiology, and the World Heart Federation, updated the 2000 consensus document (25). By 2007, these principles had been redefined by the Second Global Task Force, and accepted by the WHO, including an emphasis on clinical factors which might lead to a MI (28).

Even more recently, in 2012, the universal definition was updated for the third time by the Global Task Force. The new MI definition, described in *Table 1*, is based on clinical symptoms, biomarkers, ECG changes, cardio-imaging and angiographic data. The novel criteria underscore the importance of imaging to detect very small amounts of myocardial injury or necrosis (29). It is likely that availability of upcoming novel biomarkers able to

detect brief periods of minor ischemia will lead to a further reclassification of unstable angina pectoris as MI (30).

2.3.1.3 Treatment

Timely treatment is recommended in order to avoid cardiac damage or mortality. Invasive procedures e.g., percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABGs) have been widely incorporated into clinical practice. The preferred method for reperfusion of ST segment elevation MI (STEMI) is primary PCI and its indication is based on timing from symptom onset and the patient´s clinical status (31). In addition to lifestyle changes recommended for secondary prevention, drug therapy with angiotensin converting enzyme inhibitors, antiplatelet drugs, beta-blockers and statins has been shown to reduce complications post MI (32).

Table 1. Definition and criteria for acute MI.

Any of the following criteria meets the diagnosis for MI:
1. Rise/or fall of cardiac biomarker (preferably cardiac troponin) with at least one of the following: *Symptoms of ischemia *New or presumed new significant ST-segment-T wave (ST-T) changes or new left bundle branch block (LBB) *Pathological Q waves in the ECG *Imaging evidence of new loss of viable myocardium or new regional Wall motion abnormality *Identification of an intracoronary thrombus by angiography or autopsy.
2. Cardiac death with symptoms suggestive of myocardial ischemia and presumed new ischemic ECG changes or new LBBB, but death occurred before cardiac biomarkers were obtained, or before values would be increased.
3. Percutaneous coronary intervention (PCI) related MI arbitrarily defined by elevation of troponin values >5 times the 99th percentile URL if the pre-procedure value is normal. If baseline are elevated but are stable or falling, an increase of >20 % is required. In addition, patients should have either (I) symptoms suggestive or (II) myocardial ischemia or (III) new ischemic ECG changes or angiographic findings consistent with procedural complication or (IV) imaging demonstration of new loss of viable myocardium or new regional Wall motion abnormality are required.
4. Stent thrombosis associated with MI when detected by coronary angiography or autopsy in the setting of myocardial ischemia and with a rise/or fall of cardiac biomarker values with at least one value about the 99th percentile URL.
5. Coronary artery bypass grafting (CABG) related MI is arbitrarily defined by elevation of cardiac biomarker values (>10 x 99^{th} percentile URL). In addition, (I) new pathological Q waves or new LBBB (II) angiographic documented new graft or new native coronary artery occlusion or (III) Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality

Source: *White H, Thygesen K, Alpert JS, Jaffe A. Universal MI definition update for cardiovascular disease. Curr Cardiol Rep. 2014;16(6):492.*

2.3.1.4 General overview-Ischemic stroke

Ischemic strokes, another clinical manifestation of CVD, accounts for 87% of all strokes (33). A pro-inflammatory phenotype is a feature shared by CHD and ischemic stroke. In fact, several studies have shown that systemic inflammation profile predisposes people to ischemic cerebrovascular disease and is involved in the physiopathology of brain ischemia (34, 35).

Atherosclerosis contributes to a large proportion of strokes. Findings from a necropsy study reported that, compared to patients with other neurological diseases, atherosclerotic plaques and signs of MI were present in the coronary arteries of 72.4% and 40%, respectively, of patients with fatal stroke (36). Furthermore, some of the conventional CV risk factors such as sex, age, alcohol intake, HDL cholesterol, and physical inactivity confer similar prediction for risk of ischemic stroke (37). A strong association with the risk of stroke is observed in the presence of atrial fibrillation, prevalent stroke and hypertension, the later considered as the main risk factor for both, haemorrhagic and ischemic stroke (38).

In addition, sex differences with regards to the occurrence and aetiology of stroke have been described. Compared to men, stroke affects a bigger proportion of women due to higher longevity and onset of disease in the elderly (39). While cardioembolism is the main cause of stroke in women, large and small vessel disease is the main cause among men (40). Likewise, disability/quality of life post-stroke are poorer in women (39).

3 BIOMARKERS

A biological marker (biomarker) is "any substance, structure or process that can be measured in biospecimens and may be associated with health-related outcomes" (41). From a practical standpoint, certain characteristics such as validity, reproducibility, cost-effectiveness, and safety make a novel molecule attractive as a biomarker candidate (42). It is also essential to evaluate not only that biomarkers are independent predictors of disease, but also to distinguish if they are causally related to mechanisms underlying the disease of interest. They are categorized as biomarkers of exposure, which are used in risk prediction, and biomarkers of disease, used in screening, diagnosing and monitoring the disease progression (43).

In CVD, the clinical practice relies upon the use of diagnostic and prognostic biomarkers; however we still lack biomarkers of prediction. Up to date, a number of inflammatory biomarkers in relation to CVD have been studied and so far, these biomarkers have proven to be of limited value as supplementary strategies in CVD risk assessment (44). Furthermore, across different studies, novel biomarkers have only marginally improved the metrics of reclassification, discrimination and calibration to ameliorate risk prediction above and beyond the traditional risk factors (45, 46). For instance, the association between C-reactive protein (CRP), an inflammatory marker, with CVD has been perhaps among the most extensively explored associations in epidemiological cohorts. Although high CRP levels may indicate increased CVD risk (47, 48), the pathophysiological role of this marker in the atherosclerotic process has been questioned (49). Recent genetic data from Mendelian randomization studies failed to provide evidence for a causative role of CRP in CVD (50, 51).

Therefore, further research in the field of novel inflammatory biomarkers and their clinical validation is essential to elucidate if they can provide a better prediction of future CVE and to increase our understanding of the mechanisms in atherosclerosis related pathways (52).

Chemokines and cytokines, such as Interleukin 8 (IL8) and Interleukin 6 (IL6), have been detected in human atherosclerotic plaques and regulate mechanisms by which innate and adaptive immune cells control plaque inflammation (53, 54). It is hoped that further insights into these molecular mechanisms will lead to novel therapeutic strategies to decrease the persistent residual risk of CVD in the general population.

3.1 INTERLEUKIN 8

IL8, a chemokine circulating in picomolar concentrations, has been implicated in a wide range of acute and chronic inflammatory pathologies. IL8 is synthetized by various immunologic and nonimmunologic cells and exerts pleiotropic effects (55).

3.1.1 IL8 in atherosclerosis

IL8 participates at different stages in the initiation and development of atherosclerosis:

1. **Atherogenesis:**

   IL8 binds to its endothelial receptors *IL8R* (*CXCR1* or *CXCR2*) and acts as a chemoattractant for monocytes and neutrophils into the vascular wall (56), a pivotal step in atherogenesis (*Figure 2*).

2. **Transition from acute to chronic inflammation**

   IL8 is involved in the transition from acute to chronic inflammation (57). As shown in *Figure 3*, during the early phases of the inflammatory process, IL8 is released from endothelial cells (ECs) and neutrophils upon thrombin stimulation (58), among others, and promotes the synthesis of a monocyte chemoattractant protein 1 (MCP-1). IL8 also promotes the shedding of the soluble Interleukin 6 receptor (sIL6R) from cells that express it (e.g., hepatocytes and leukocytes).

   The sIL6R then binds to the Interleukin 6 (IL6) forming a complex which binds to the membrane bound glycoprotein 130 (gp130) and the intracellular signalling begins. The soluble form of gp130 (sgp130) is circulating after shedding or proteolysis of the gp130. The sgp130 has the ability to inhibit the complex sIL6R-IL6.

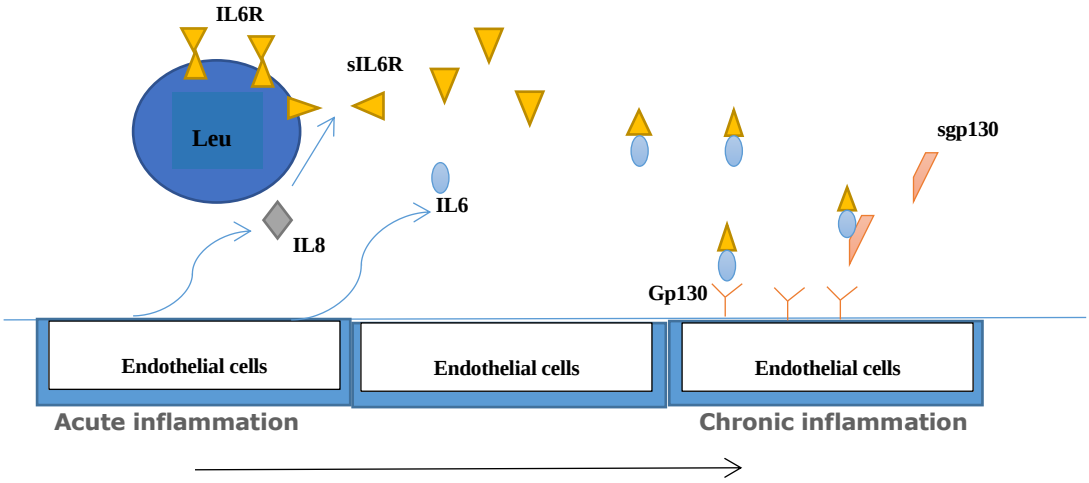


Figure 3. *IL8 in the transition from acute to chronic inflammation. Leu: leucocytes.*

3. **Atherosclerotic plaque destabilization**

   IL8 promotes an imbalance between metalloproteinases and metallopeptidases due to the diminution of the level of tissue inhibitors of metalloproteinases (TIMP-1) triggering local extracellular degradation in the plaque fibrous cap (59).

4. **IL8 in thrombogenesis**

   IL8 has been shown to increase production and surface expression of tissue factor, an important inducer of blood coagulation in monocytes (60) providing a potential link between inflammation and thrombosis.

5. **Cardiac remodeling after MI**

   IL8 promotes angiogenesis (61), inhibits apoptotic response and therefore enhances ECs survival (62). IL8 mobilizes progenitor ECs from the bone marrow, which promote neovascularization (63). In experimental models of ischemic injury, high circulating IL8 may be an adaptive response of the myocardium for repair and revascularization to maintain oxygen supply after hypoxia/ ischemic injury (64).

3.1.2 IL8 isoforms

During the 90´s, a more complex role for IL8 in the inflammatory process was recognized, yet not fully addressed. IL8 circulates in two major isoforms: a 72-aminoacid monocyte-derived form ($[Ser\text{-}IL8]_{72}$) and a 77-aminoacid ($[Ala\text{-}IL8]_{77}$), mainly produced by ECs (65). These two isoforms might be produced under different stimuli, at different times during the development and mediate different effects (66) . Whereas the $[Ser\text{-}IL8]_{72}$ isoform has been shown to be more potent in stimulating neutrophils in *in vitro* assays (67), $[Ala\text{-}IL8]_{77}$ may attenuate inflammatory events at the interface between the blood and the vessel wall and has been shown to exert a cardioprotective effect in animal models of ischemia and reperfusion (68).

The previous studies have prompted the hypothesis that IL8 affects the inflammatory process in a time dependent fashion- as pro-inflammatory in the early stages and as less inflammatory at a later stage (69). The change in activity is likely to be mediated by a switch between the two circulating isoforms (66). However, it has also been reported that the anti/pro inflammatory properties of IL8 are not only correlated to the different effects of the circulating isoforms, but also to extrinsic factors such as the directionality of local concentration gradients and the proteolytic activity in the tissue microenvironment (70). More

recently, antirombin III has been shown to inhibit the conversion of $[Ala\text{-}IL8]_{77}$ isoform to shorter forms (71).

3.1.3 Circulating levels of IL8 and risk of CVD

Few observational studies have evaluated the association between IL8 and CVD. The first study to report an association was the *EPIC-Norfolk,* a prospective population-based study, where elevated concentrations of plasma IL8 were associated with an increased risk of future CHD (72). Shortly after, an analysis performed in the population-based *MONICA/KORA Augsburg* cohort reported an association between IL8 and incident CHD independently of the conventional CV risk factors (73). However this association is attenuated in their final model after adjustments for CRP and IL6.

Other researchers have reported associations of IL8 with risk of CVD in populations with established CVD. IL8 predicted secondary CVE after 7 years of follow-up independently of other cytokines and CRP in patients with CHD (74). CVE was defined as CV death, non-fatal MI, angina pectoris, repeated revascularization after PCI or CABGs, heart failure, non-fatal cerebrovascular events and arrhythmia. In a sub-study derived from the *CORONA* trial, elevated levels of IL8 were associated with adverse outcomes in a population with chronic heart failure (CHF) where the primary endpoint was the composite of CV mortality, non-fatal MI or non-fatal stroke (75).

3.2 GENETIC EPIDEMIOLOGY OF CVD

The individual susceptibility to CVD is determined by an interaction between genetic and environmental exposures. Variability of a particular phenotype may be a consequence of genetic variation in individuals exposed to the same environment, and likewise, of different environmental milieu in individuals with the same genotype (76).

Genetic epidemiology has been helpful in defining the degree of the genetic contribution to CVD from a population perspective. Heritability of CV death ranges from 50-60% among males and 33-55% among females according to twin studies, particularly at younger ages (77, 78). On the other hand, the presence of some conditions such as elevated BMI, seem to reduce the influence of genetic variants on CVD risk, thus indicating that genetic factors participate more actively in the pathogenesis when other conventional CV risk factors with a genetic and an environmental component are absent (79).

Genome wide association studies (GWAS) have allowed the identification of genes contributing to atherosclerotic CVD. Data obtained from the collaboration among several research groups have shown associations of single nucleotide polymorphisms (SNPs) with risk of CVD [(80, 81) among others].

As represented in *Figure 4*, further interest has arisen in investigating if (1) genetic variants associated with the risk of CVD are also (2) associated with levels of circulating biomarkers, given that (3) circulating biomarkers under investigation are associated with the risk of CVD. As summarized by the gray line (4), this approach, the so-called Mendelian randomization, has been discussed as a way to assess causal effects of a biomarker with the risk of CVD.

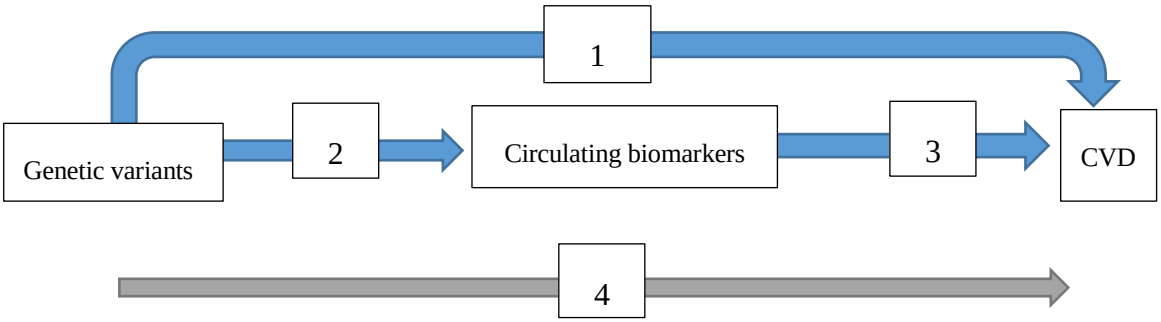


Figure 4. *Mendelian Randomization approach*

In order for the Mendelian randomization to hold, some assumptions are needed, such as that the genetic variant cannot affect the outcome through pathways other than through the circulating biomarkers (82) and that the interpretation may hampered if the genetic variant has pleiotropic effects (83).

3.2.1 *IL8* genetic variants and risk of CVD

The *IL8* gene maps on the chromosome 4q13-q21, while the *IL8R* gene maps on chromosome 2q34-q35. Few studies have explored the association of polymorphisms in the *IL8* gene with risk of CVD. In a case-control study, Vogiatzi and colleagues evaluated the -251 A/T variant in the promoter and the 781 C/T intronic polymorphism of the *IL8* gene in patients with CHD, where CHD was defined as angiographic evidence of at least one pericardial coronary artery with more than 70% stenosis (84). The control group were age matched subjects without evidence of CHD, but at least one CV risk factor for CHD. They reported no association of these polymorphisms with risk of CHD. However, they found that in the group

with CHD, individuals with the minor allele (AA) at the -251 A/T SNP as well as the combined genotype $AA_{251}TT_{781}$ were associated with a reduced risk of ACS.

An opposite finding was reported in a study of a Chinese Han population where the minor allele at the -251 A/T SNP was associated with a 30% increase risk of ACS in individuals with established CHD, defined as having a stenosis (≥50%) in at least one major coronary artery branch (85). Yet, the replication population, recruited from the same region using the same criteria, showed an inverse association with the risk of ACS.

The association of the gene coding *IL8R* (*CXCR1* and *CXCR2*) with risk of CVD, to our knowledge, has not been reported.

3.3 BEYOND IL8: THE INTERLEUKIN 6 CYTOKINE FAMILY

Interleukin 6, a cytokine involved in a wide variety of pathologies, has inflammatory and anti-inflammatory properties. In the setting of CVD, IL6 participates in the oxidation of lipoproteins, recruitment and activation of pro-inflammatory cells as well as in the pro-coagulant activities (60, 86). Several cohorts and case-control studies have described an association between circulating IL6 levels with increased risk of MI (87, 88), all-cause and CV mortality in the elderly (89), as well as with the progression of atherosclerosis in patients with vascular risk factors (90).

However, the association of other novel members of the IL6 family, sIL6R and sgp130, with risk of CVD has been less studied.

3.3.1 Soluble Interleukin 6 receptor

IL6 can bind to a membrane-bound receptor or to a soluble receptor, each one of them triggering signals with selective effects (91). When IL6 binds to the membrane bound IL6R, a pathway named *classic signalling*, the initial immune response and induction of acute phase proteins in hepatocyte is triggered. This signalling is limited since this receptor is only present in hepatocytes and in immunological cells (neutrophils, monocytes/macrophages and some lymphocytes) (92).

When IL6 binds to the sIL6R, the complex IL6-sIL6R binds to gp130 expressed in all human cell surfaces and initiates the so-called *trans-signalling* pathway, (93). In this way, the response to IL6 is expanded to other cells that do not express the IL6 membrane bound receptor. The sIL6R can be generated by alternative splicing of the IL6R mRNA and by shedding of the membrane bound receptor, which accounts for 90-99% of the circulating sIL6R (94). This pathway may be more crucial for the pro-inflammatory effects of IL6 (95).

Recently, genetics consortia have provided evidence for a causal role of the IL6R related pathways in CHD. A genetic variant causing an Asp358Ala aminoacid change in the membrane-bound of the IL6R was shown to be associated with a slightly increased risk for CHD (80) as well as with a reduced risk for CHD (51, 96, 97). The minor allele was associated with decreased concentrations of fibrinogen, CRP and with increased levels of sIL6R. This mutation constitutes the major determinant of the circulating sIL6R (98) and has been shown to reduce shedding of the IL6R up to fivefold (99). These findings suggest that, in the presence of circulating IL6, this-genetic-variant-incrementing-sIL6R favours the

trans-signalling pathway; and thus underscores the importance of sgp130 levels as responsible for the adequate control of IL6 *trans-signalling*.

3.3.2 Soluble glycoprotein 130

Sgp130 acts as an inhibitor of the IL6-sIL6R complex, and therefore prevents its binding of the complex to the gp130 receptor. IL6 binds to the sIL6R with an affinity of around 1nM whereas the complex of IL6 and IL6R binds to gp130 with a 100 times higher affinity (95). As a result, IL6 binds to sIL6R and the IL6/sIL6R complex will be immediately neutralized by sgp130. Thus IL6 in the circulation is buffered by the sIL6R and the complex IL6-sIL6R is buffered by sgp130 (100).

Recently, an experimental study in mice has reported a significant decrease in the development and progression of atherosclerotic plaques with pharmacological inhibition of the IL6 *trans-signalling* using the fusion protein sgp130Fc (101). In fact, the first human phase I study with sgp130Fc is ongoing in chronic inflammatory diseases (100).

Elevations in sgp130 serum levels have been associated with all-cause and CV mortality, in elderly patients with CHF of ischemic aetiology (102). As well, a descriptive, small hospital-based pilot study reported higher serum levels of sIL6R in acute MI and CHD patients as compared to controls but no differences in the sgp130 serum levels in the three groups (103).

4 AIMS

The overarching aim of this thesis was to investigate the association of inflammatory biomarkers with risk of CVD with the final scope to identify potential novel predictors of incident CVE.

The specific aims were as follows:

1. To study the association between levels of IL8 and the genetic variants at the *IL8* and *IL8* receptor gene with the occurrence of MI.
2. To identify novel genetic variants associated with levels of circulating IL8.
3. To study the association of serum levels of IL8 with the risk of incident CVE.
4. To investigate the association between the sIL6R and sgp130 with the occurrence of MI.

5 METHODS

5.1.1 The SHEEP study

Results derived from this thesis (study I, II and IV) are based upon data obtained from the Stockholm Heart Epidemiology Program (SHEEP), a population-based case-control study conducted in Stockholm County. The study base comprised all Swedish citizens resident in the Stockholm County from 1992-1994 who were 45 to 70 years of age and were free of previous clinically diagnosed MI.

5.1.1.1 Cases

Incident non-fatal MI cases in the study base were continuously identified at 10 emergency hospitals within the county of Stockholm. Diagnosis of MI was based on two of the following criteria (i) clinical history (ii) augmented specified levels of the enzymes creatine kinase and lactate dehydrogenase and (iii) specified ECG changes (pathologic Q-wave in at least two ECG leads), according to the Swedish Association of Cardiology in 1992/1994 (104). Male cases were identified during a two year period (1992-1993) whereas female cases during three years (1992-1994). If cases survived at least 28 days post MI, they were classified as non-fatal; otherwise, they were considered fatal cases. A larger proportion (97%) of hospitalized MI cases were treated in internal medicine departments where one cardiologist and 2-4 nurses per hospital were responsible for identifying study participants. Identification of the rest of the cases treated in other departments (surgery, geriatrics, nephrology, neurology, pneumology) was done through the computerized hospital discharge register.

During the period January-October 1992, the upper age limit was 65 years; from 1 November 1992 and onwards it was 70 years.

5.1.1.2 Controls

Controls were randomly sampled from the study base and identified through the computerized register of the Stockholm County population, using density sampling and matching for sex, age (5-year interval) and hospital catchment area. This selection of controls was done within 2 days of the case occurrence. Five control candidates were sampled at the same time, so that a potentially non-responding control could be substituted by a different candidate who belonged to the study base at the time of the case occurrence. Due to some late response for the initial control, both the initial and a substitute control were included sporadically. Consequently, more controls than cases were recruited.

A total of 5452 subjects were enrolled in the study (2246 cases and 3206 controls). Non-fatal cases had a participation proportion of 87% (n= 1643) while controls had a participation proportion of 73% (n= 2339).

Using the Stockholm County National Patient Register, both, case and control candidates were investigated for previous MI (ICD9-codes 410 or 412 or corresponding codes in previous ICD revisions) since 1975.

The results of the studies using the SHEEP material in this thesis are restricted to non-fatal cases, who volunteered to give a blood sample and had no further MI before blood sampling (n=1213), and their sex, age and their hospital catchment area matched controls (n=1561). Fatal cases (n=603) were excluded as the subjects did not provide blood samples.

Clinical investigations were performed on average three months after the onset of disease (to allow the patients to regain metabolic stability) including collection of overnight fasting samples (whole blood for DNA extraction, serum, EDTA and citrated plasma). The serum samples were separated within 1 hour and kept frozen at -70° C until analysed. The DNA was stored at -80° C.

5.1.1.3 Exposure information:

Postal questionnaires were distributed to non-fatal cases and controls, where extensive information on a variety of potential CV risk factors was collected. Unless the subject declined to participate, up to four reminders could be given if necessary. In addition, a complementary telephone interview was carried out to complete missing information.

Participants were invited for health examinations that took place at the outpatient clinics of the 10 emergency hospitals. Controls undertook examination as close as possible to the date of the cases to avoid biases due to seasonal variation with regards to blood parameters. Anthropometric measurements such as BMI; (Kg/m^2), systolic and diastolic blood pressure (SBP and DBP; mmHg) were assessed. Biochemical analyses were done using radioimmunoassay (RIA) kits [insulin (µU/L)], enzyme-linked immunosorbent assays (ELISA) [von Willenbrand factor, IL6], enzymatic colometric methods [total cholesterol (mmol/L), triglycerides, HDL cholesterol], immunochemical techniques [ApoA1 and ApoB1 (g/L)], RIA techniques [insulin growing factor binding protein 1 (IGFBP1; mg/L)], fibrin polymerization test according to method described by Vermylen et al. (105) [fibrinogen (g/L)], spectrolyze PAI-kit [plasminogen activator inhibitor (PAI-1; mg/L)], high sensitive

immunoephelometric assay [CRP (mg/L)], serum glucose, blood lipids, Quantikine HS human TNF-α [tumour necrosis factor-α (TNF- α; ng/L)].

5.1.1.4 Ethics

The SHEEP study was carried out in accordance with the Helsinki Declaration and was approved in 1991 by the Regional Ethical Review Board at Karolinska Institutet. Participants gave their oral informed consent since at the time of enrolment, no written informed consent was currently in use.

5.1.1.5 Serum measurements

5.1.1.5.1 IL8

After thawing, 25 µL of the 1847 available serum samples (1133 controls and 714 cases) were measured for IL6 and IL8 concentrations using an electrochemiluminescence immunoassay plates from Meso Scale Discovery´s Multi-Array ® (MSD). Two sets of batches were analysed in 96 Well 4-Spot using MSD® 6000 instrument (n=1128 samples) and MSD SI2400® instrument (n=719 samples), following the manufacturer´s assay and analysis protocols. To anchor between the two batches, new and old calibrators were run on the same plate and at the same time. The limit of detection for each plate was determined based on the linearity of the standard curve following the manufacturer´s instructions. MSD appears to have a better sensitivity at lower concentrations for IL8, as compared to other analytical platforms (106).

Duplicates (intra-intervariability) were measured in approximately 40 % of the samples and results were recorded as the average. Samples with coefficient of variation (CoV) higher than 25 (n=37) were excluded from the analyses. The intra-and interassay CoV were (%) 7.9 and 8.0 respectively.

According to the number of times being thawed, samples were recorded as never been defrosted (n=1723), defrosted once (n=121) and defrosted twice (n=3). In order to determine if frequency of thawing influenced levels of IL8, n= 104 samples were measured repetitively to compare IL8 levels between samples that were never thawed and samples defrosted twice. Reproducibility was evaluated using the Spearman correlation coefficient. Correlation between the two measurements was 0.61.

As added measurements of accuracy, levels of IL6 in serum were correlated to the IL6 levels measured earlier in the SHEEP by enzyme-linked immunosorbent assays (ELISA) (87). The two methods replicated fairly well.

5.1.1.5.2 sIL6R

Serum concentrations of sIL6R were measured using the MesoScale Discovery (MSD) Human Cytokine Assay (Gaithersburg, MD, USA), in available serum samples, following the manufacturer's assay protocol. In total, 1785 serum from study participants (682 cases and 1103 controls) were measured. Samples were diluted 1:75 and concentrations were derived from the standard curve and are expressed in nanograms per milliliter (ng/mL). The minimum detectable level for sIL6R was 0.1 pg/mL.

To calculate the intra variability of the assay, n= 267 samples for the sIL-6R were run in duplicate. In order to assess the inter assay variability n= 67 samples of the sIL-6R were run in independent experiments. The intra-and interassay CoV were 6.1 % and 3.8% respectively.

5.1.1.5.3 sgp130

Due to absence and/or scarcity of serum, a total of 1726 serum samples (664 cases and 1062 controls) were measured in serum for concentrations of sgp130. We used an assay from R&D systems®, Quantikine® ELISA according to the protocol instructions. Samples required a 100-fold dilution. To calculate the intra and intervariablity, n=25 samples were duplicated intra-plate, whereas n=37 samples were duplicated in independent experiments. Serum concentrations were derived from the standard curve and are expressed in ng/mL. Intra and intervariability of the plates were 1.8 and 12.1 % respectively. The minimum detectable amount of sgp130 was 0.08 ng/mL.

5.1.1.6 Genotyping:

One tag SNP at the *IL8* (**rs2227306**C/T) which lies in the first intron of the gene and three tag SNPs in the *IL8* receptors gene (**rs6723449**T/C, **rs1008563**C/T, **rs4674258**C/T) were genotyped by MALDI-TOF (Matrix Adsorbed Laser Desorption-Ionisation-Time Of Flight) on Massarray analyser platform, after their identification using HapMap database. The numbers of subjects with genotype available were: *IL8* SNP (1102 cases and 1434 controls), *IL8* receptor **rs6723449** (1154 cases and 1501 controls), *IL8* receptor **rs1008563** (1134 cases and 1465 controls), *IL8* receptor **rs4674258** (1143 cases and 1477 controls).

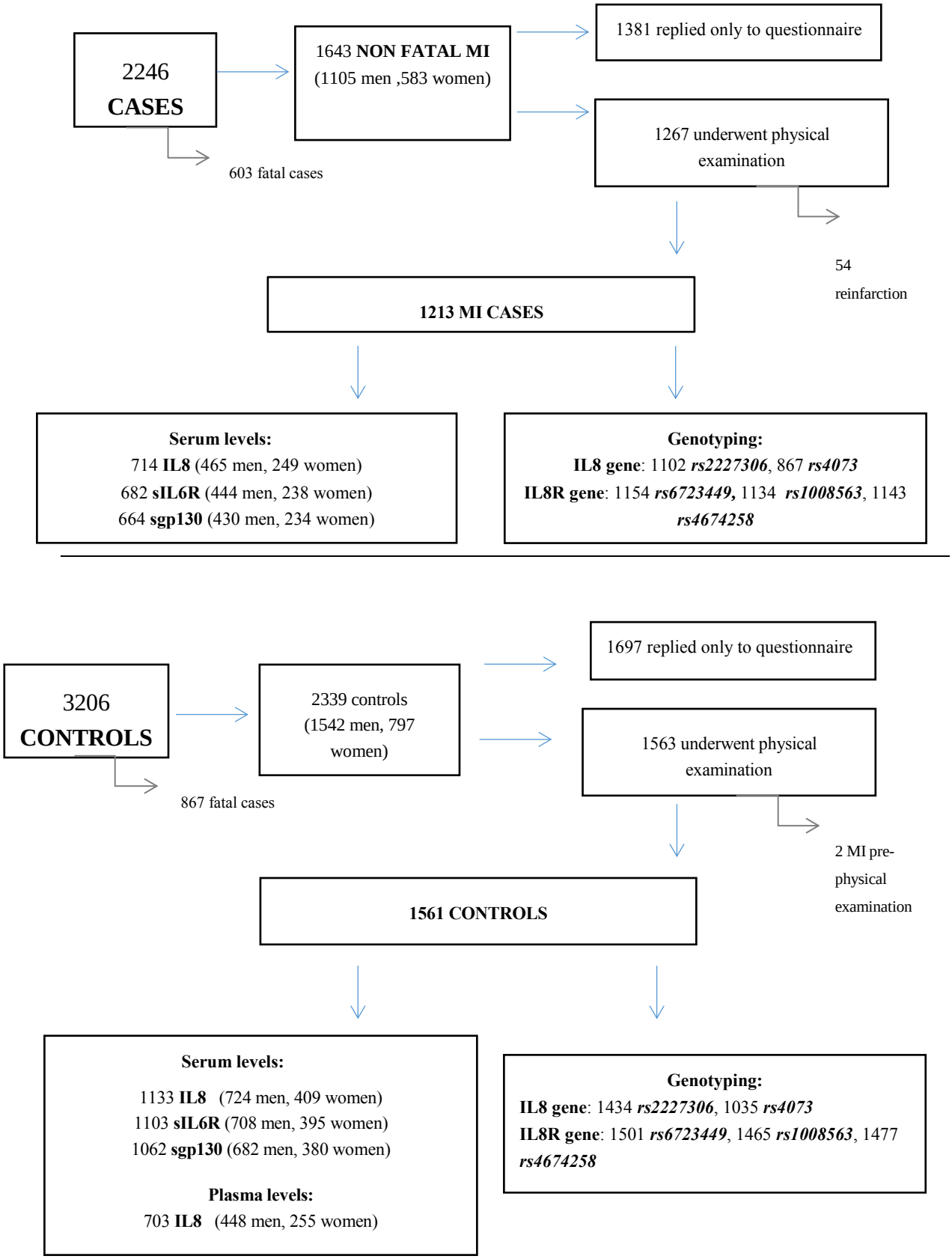
In addition, we have genotyped the promoter variant **rs4073**A/T, in position -251 in the *IL8* gene. This SNP was genotyped with a custom made assay purchased from Applied Biosystem (ABI) and amplified with the TaqMan Universal PCR protocol. Unfortunately, a fraction of the samples genotypes were missing as a result of failures in the DNA reading. In total, n= 867 cases and n= 1035 controls were genotyped for rs4073.

5.1.1.7 Illumina iSelect CardioMetabo200kb

Genomic DNA from the SHEEP study participants was genotyped using the Illumina iSelect CardioMetabo200kb (CardioMetabochip), a custom- made genotyping array that captures DNA variation at regions identified by meta-analyses of GWAS for diseases and traits relevant to metabolic and atherosclerotic/cardiovascular diseases. The array contained approximately 200,000 variants mapping at genomic region of potential relevance for these traits, identified by chromosomal location and/or SNP identification number (107).

More detailed information on number of participants and selection criteria for Studies I-II and IV is presented in *Figure 5*.

Figure 5. **Flowchart of the recruitment of participants in the SHEEP study.**



5.1.2 Cohort of 60 years old Men and Women from Stockholm

Results from Study III, were derived from the cohort of 60 years old Men and Women (60YO). The study base comprises men and women born between 1 July 1937 and 30 June 1938 who were living in the Stockholm County between August1997 to March 1999.

Every third man (n=2779) and women (n=2681) who turned 60 during the time period of recruitment were randomly selected from the Total Population Register in Sweden and invited to participate in a health screening for CVD. An invitation letter was sent by mail to eligible subjects informing them about the study. In this letter, it was asked kindly to contact a booking centre to inform whether he or she would like to participate or not. The nurses working at the booking centre documented each reply. A reminder was sent to those who did not reply to the first letter.

In total 5460 subjects, 2779 men and 2681 women, were invited, out of which 4232 individuals participated; 2039 men (73%) and 2193 women (82%). The overall participation proportion was 78%.

Yearly, the cohort is matched with the Swedish National Inpatient Register and the Cause of Death register in Sweden. Through linkage with the national registers, 491first time (incident) ischemic CVE were recorded up to December 31st 2012. The composite endpoint was defined according to the International Classification of Diseases 10th Revision codes as fatal and non-fatal MI (I21, I25), fatal and non-fatal ischemic stroke (I63-I66), and hospitalization due to angina pectoris including PCI and CABGs procedures (I20, Z95.5 and Z95.1).

Individuals with previous CVE (angina, stroke and MI) at baseline (n=369) and those who did not fill in the questionnaire (n=122) were excluded, leaving a total number of 3741 study participants (1990 women and 1751 men). Exclusion was assessed through 1) the Swedish National Inpatient Register and by 2) having reported in the questionnaire a previous MI, stroke, heart failure and/or angina /claudication.

The study has been approved by the Regional Ethical Review Board at Karolinska Institutet, Stockholm, Sweden. All study participants gave their oral consent to be enrolled in the study, since written informed consent was not in current use at the time this study was conducted.

5.1.2.1 A nested case- control design

For study III, a nested case-control was designed. For each incident CVD case, two controls were randomly selected from the cohort, with the exception of 1 male participant where only 1 control could be selected. Selection of controls was done randomly from the study base

continuously over time (density sampling) and matched for sex and time at risk (those controls who were not classified as a case +/- 60 days at the date of the event) and who were still alive.

More detailed information about number of participants and selection criteria for Study III is shown in *Figure 6*.

5.1.2.2 Exposure information

A comprehensive questionnaire regarding a wide range of exposures was distributed via mail and filled in by the study participants. If needed, a study nurse assisted with filling in the questionnaire. All participants underwent a thorough physical examination including anthropometrical tests and collection of ECG. Venous blood samples, including serum and plasma, were drawn from an antecubital vein after overnight fasting 24:00 the night before and stored at biobanks (-80 C).

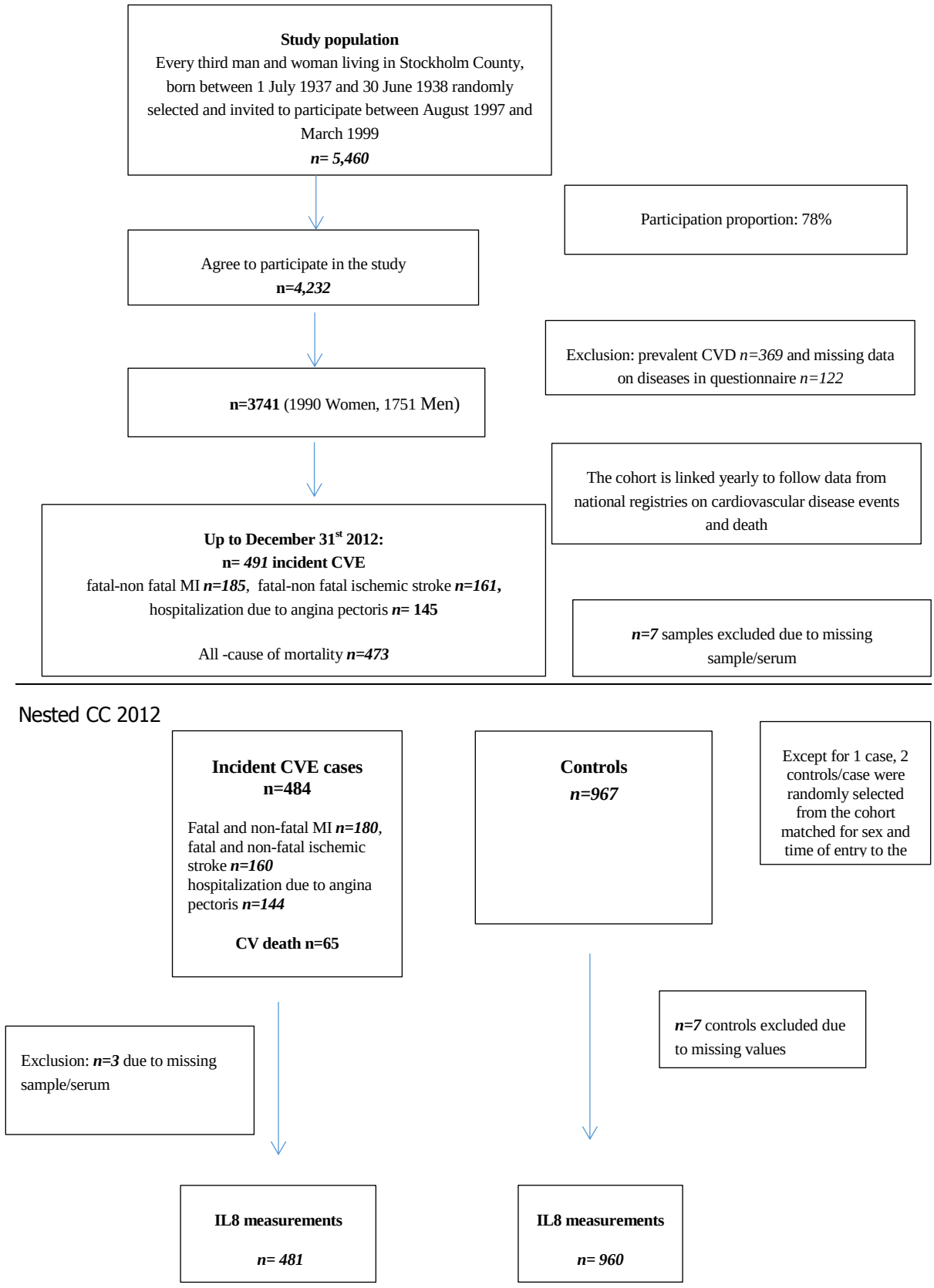
Blood pressure (systolic/diastolic) was measured twice after 5 minutes of rest and the mean value was calculated. BMI was computed from height and weight measurements and expresses in Kg/m^2. Cholesterol and triglyceride levels in serum were analysed using enzymatic methods (Bayer Diagnostics, Tarrytown, NY, USA). Serum glucose was measured with an enzymatic colorimetric test (Bayer Diagnostics) (108).

5.1.2.2.1 IL8 measurements

Serum samples were retrieved from frozen storage, thawed, and IL8 levels were measured using electrochemiluminescence immunoassay multiplex plates produced from Meso Scale Discovery´s Multi-Array ® (MSD). Serum concentrations were derived from the standard curve and expressed as picograms per millilitre (pg/mL). Together with IL8, other cytokines such as IL6, IL1 β and TNF-α were measured. Samples were analysed in a random order and blinded with regards to case-control status in order to avoid systematic bias.

To calculate the intra and inter assay variability, n= 68 samples were run in duplicate and n=32 samples were run in independent experiments respectively. Intra and intervariability of the plates were 9.9% and 9.7%, respectively.

Figure 6. Flowchart of the recruitment of participants in the Cohort of 60 years old Men and Women from Stockholm**.**



5.1.3 Replication cohorts

In Study II, the genetic associations observed in the SHEEP material required replication in independent studies. Therefore, the studies initially contacted were those where we could replicate our findings *in silico*, i.e., IL8 measurements were available and the same genetic variants had been genotyped.

Our results were replicated in three independent populations derived from:

5.1.3.1 The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) cohort:

The cohort was established to investigate the predictive power of different measurements of endothelial function and arterial compliance in the elderly (109) . The study base comprised all subjects aged 70 years living in the community of Uppsala, Sweden from 2001-2004.

Eligible subjects were chosen from the register of community living and were invited to participate in the study in a randomized order. Of the 2025 subjects invited, 1016 subjects agreed to be enrolled in the study (participation proportion of 50.1%). Participants were asked to answer a large questionnaire concerning their medical history, lifestyle factors and medication. Anthropometrical measurements, blood pressure and blood sampling was also undertaken at baseline. Sampling were drawn after an overnight fast and stored at -70 ° C until analysis.

The study was approved by the Ethics Committee of the University of Uppsala and the participants gave informed consent.

5.1.3.1.1 *IL8 measurements*

IL8 was measured on serum using Evidence array biochip analyser (Randox Laboratories Ltd, Crumlin, UK) (110). The functional sensitivity for IL8 was 1.5 pg/mL. From the 1016 study participants, 13 individuals were excluded due to missing values on IL8, leaving 1003 study participants. For our analysis, IL8 values were log transformed, and values =0 were excluded from the analysis, therefore n= 931 participants were included.

Participants of the PIVUS study were genotyped using the CardioMetabochip.

5.1.3.2 The Stockholm Coronary Artery Risk Factor (SCARF) study

The SCARF study is a MI case-control study from the northern part of Stockholm designed to investigate novel biochemical and molecular genetic risk markers for CAD. From January 1996 to December 2000, all individuals aged <60 years who were admitted for acute MI to

the coronary care units of the three hospitals (Danderyd, Karolinska and Norrtälje Hospital) in the northern part of Stockholm were considered for inclusion in the study (n=755). Exclusion criteria were those with previous MI (n=102), diabetes mellitus type 1 (n=23), renal insufficiency (n=9), any chronic inflammatory disease (n=13), drug addiction (n=10), psychiatric disease (n=3) or unwillingness to participate in the study (n=19). In addition 143 participants were excluded due to psychosocial reasons, leaving a total number of 433 participants. Out of those, individuals were excluded due to program incompleteness (n=51), withdrawn consent (n=19), comorbidities (n=12) or protocol violation (n=7), and deaths (n=3). This resulted in 387 first time MI cases. One control was randomly recruited from the general population via the Total Population Register matched on sex, age and residential area. About 3 months after the onset of MI, study participants were interviewed regarding medical history and lifestyle factors. Collection of whole blood for DNA extraction and plasma were collected under fasting conditions (111).

The study was approved by the Ethics Committee of the Karolinska University Hospital and the participants gave informed consent.

5.1.3.2.1 *IL8 measurements**:***

Fasting plasma concentrations of IL8 were determined using Evidence® array biochip analyser (Randox laboratories ltd., Crumlin, UK) (110). IL8 was measured in n= 361 cases and n= 374 controls. In order to avoid the potential influence of the case status on IL8 levels, only controls with available IL8 measurements (log transformed) and with genotype were considered for analysis (n=350).

Participants of the SCARF study were genotyped using the Cardiometabochip.

5.1.3.3 The Malmö Diet and Cancer study-Cardiovascular cohort (MDC-CC)

The Malmö Diet and Cancer study is a prospective population-based cohort consisting of 30,447 individuals. Eligible participants comprised all men, age 46-73 years and women, age 45-73 years, resident in Malmö from 1991 to 1996. Participants were invited to fill out a questionnaire as well as to undergo physical examination with blood sampling.

Furthermore, a total of 6103 men and women were randomly invited to participate in the cardiovascular arm of the population- based MDC cohort, the MDC-CC, which is a sub-study of the epidemiology of CVD (112). After 15 years, during 2007-2012, re-examination and blood sampling were performed in a subset of individuals (n=3300) that agreed to do so. All

participants provided written informed consent and the study was approved by the Regional Ethical Review Board in Lund, Sweden.

5.1.3.3.1 *IL8 measurements*

Circulating levels of IL8 were measured at baseline in heparin plasma by an electrochemiluminescence immunoassay (Human Ultra-Sensitive Kit) using a SECTOR Imager 6000 instrument (MesoScale Discovery, Gaithersburg, MD, USA). Levels of IL8 in plasma were measured in close to 700 individuals (n=696) randomly selected from the cardiovascular arm of the MDC cohort. In addition, close to 800 individuals (n=797) with metabolic syndrome according to the updated NCEP III definition, were selected for IL8 measurements.

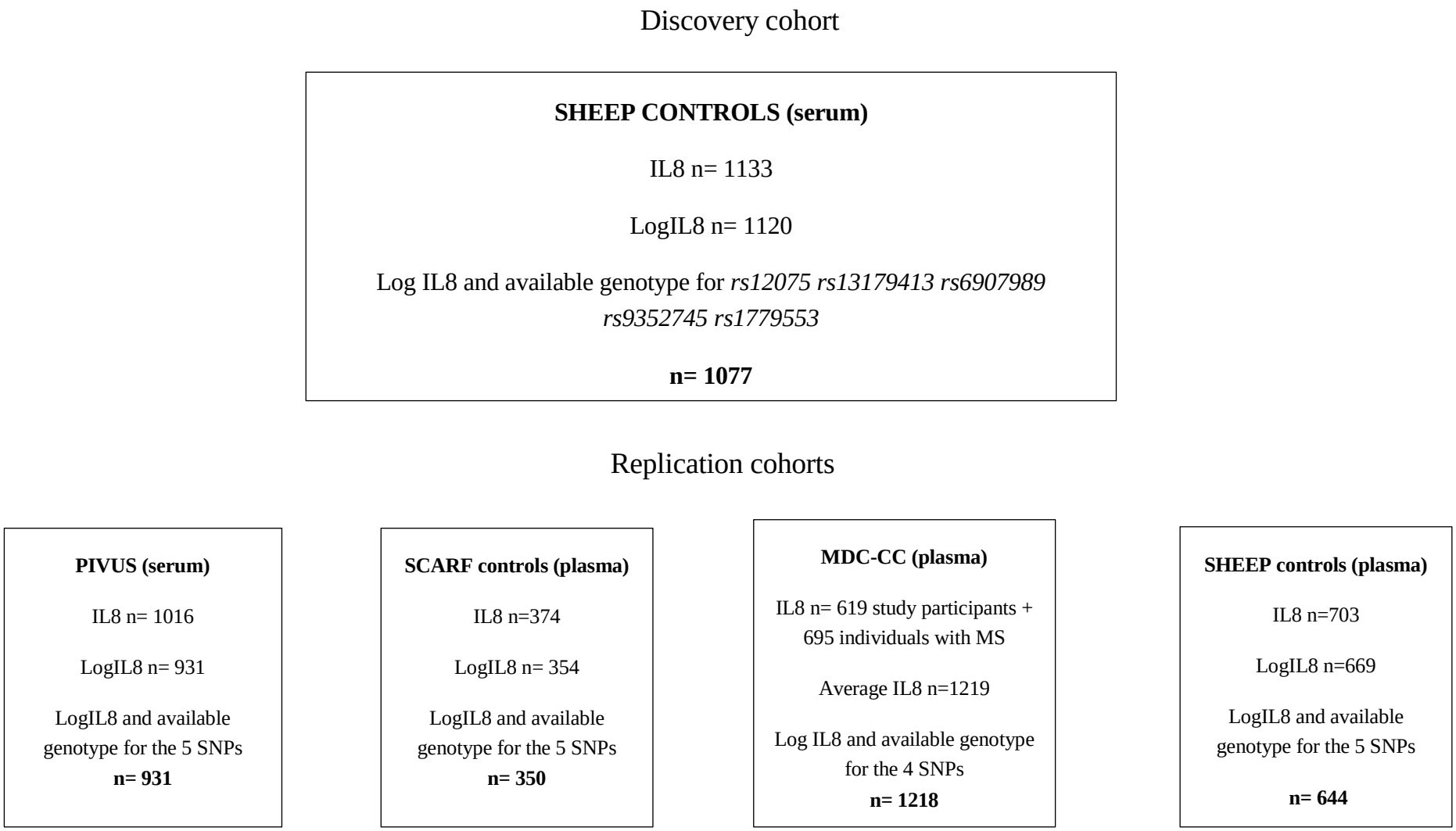
We have used both measurements and calculated the average in the presence of duplicates. The duplicates were both measured in the baseline sample obtained at the same time.

We received data for n=619 participants and n= 695 individuals with metabolic syndrome. A total of n=1218 study participants with IL8 measurement and genotype available were included.

Participants of the MDC-CC study were genotyped using Illumina's OmniExpress chip.

More details about the study participants for Study II are described in *Figure 7.*

Figure 7. Flowchart of the recruitment of participants in the discovery and replication cohorts reported in study II



5.2 STATISTICAL ANALYSES

The measured biomarkers were not normally distributed; therefore non-parametric methods (Kruskall Wallis test) were used throughout the four studies. The intravariability of the test was calculated directly from the MSD results and/or based on the average of all the standard deviations divided by the mean of the repeated values. A coefficient of variation >25% was considered as high according to the protocol. The intervariabiliy of the plate was calculated using the same formula.
When a coefficient higher than 25 was obtained, samples were repeated. If the coefficient of variation persisted, then samples were excluded from the analysis.

Due to missing data in biomarkers and genotyping, some matching pairs in the case-control design were broken. Thus, we used unconditional logistic regression as the primary analysis in Studies I, II, and IV. Conditional logistic models were also evaluated yielding similar point estimates with wider confidence intervals. Odds ratios (OR) were interpreted as incidence rate ratios (IRR) (Studies I-IV), since controls were randomly sampled from the study base using density sampling.

5.2.1 Study I

Unconditional logistic regression analysis was used to estimate the OR (the odds of exposure among the cases divided by the odds of exposure among the controls). Because of unconditional models, we have adjusted in the crude model for the matching variables in the SHEEP design: hospital catchment area and age (5 years interval). Sex was also a design variable, but results reported were sex specific. Further adjustments were done by factors considered as potentially confounding and for the traditional CV risk factors.

The models were adjusted for hypertension, defined as individuals on antihypertensive drug therapy or with a blood pressure ≥140/90 mmHg based on measurements at physical examination (mean from two readings in supine position after 5 minutes of rest) or with a history of regular antihypertensive drug therapy during any part of the last 5 years (data from questionnaire). Diabetic participants were those with a B-glucose value > 6.7 or controlling diabetes with insulin and /or other treatment at physical examination. The cut-off for hypercholesterolemia was a total cholesterol ≥ 6.46 mmol/L or receiving any lipid lowering medication. Current smoking was defined as actively smoking in the two years preceding MI. BMI (kg/m^2) was calculated from measurements at physical examination, CRP (mg/L) and insulin (μU/mL) were included as continuous variables. Drug therapy

with statins, β-blocker and hormone replacement therapy (HRT) for women, were included as reported by questionnaire.

For the SNPs association analysis, genetic effects of the three genotypes on the MI risk were unknown, thus models were constructed under the additive, dominant and recessive model of inheritance (113).

In the recessive model, individuals carrying the homozygous minor allele (aa) were considered as exposed, and therefore compared to carriers of homozygous for the major allele (AA) and heterozygous group (Aa) (two minor alleles are necessary to predict the outcome). In the dominant model, the assumption is that one minor allele is enough to predict the outcome, so carriers of the minor allele [homozygous (aa) and heterozygous (Aa)] were considered as exposed and compared to those homozygous for the major allele (AA). Finally, the additive model, considered that the effect of the heterozygote genotype (Aa) is in between the effect of the homozygote minor genotype (aa) and homozygote major genotype (AA).

OR together with 95% CI were calculated, by means of unconditional logistic regression models, to evaluate the association for each SNP with MI matched on sex, age and residential area. Further adjustments for variables related to known CVD risk factors and inflammation were performed, though not with the intention to evaluate confounding effects but rather to investigate if the genotype-MI associations observed could be mediated by metabolic phenotypes.

The analyses of the Hardy- Weinberg equilibrium were performed using the Chi-square test. Haplotypes counts and frequencies for the *IL8* and *IL8R* genes were inferred from original genotype data and the association with MI was calculated using the program THESIAS software version .3.1(114).

5.2.2 Study II

From all the SNPs contained in the Cardiometabochip, only autosomal SNPs were included. Thus, n=116 SNPs in Chr 23 and n=119 mitochondrial SNPs were excluded as part of the data cleaning procedure.

Quality control was based on the following exclusion criteria: genetic variants with minor allele frequency (MAF) < 0.001 (n = 61,153 SNPs), since rare variables may hamper the model construction, and as a general rule as the MAF increases, so does the power (115). Highly significant deviation from Hardy-Weinberg equilibrium $p<1x10^{-5}$ led to exclusion of (n=1180 SNPs), variants with > 5% missing information due to missing information and

genotype call rates (n=3475 SNPs). Thus, a total amount of 121,445 SNPs were further considered for the analysis in this study.

A Wald test was used to explore the association of 121,445 SNPs with log transformed IL8 in the SHEEP controls. The Wald test resembles a linear regression when applied to quantitative traits. This stage 1 analysis was performed using PLINK software (version 1.07) (116). The genotypes for the five statistically significant SNPs who reached the pre-defined threshold value, defined as $1 \times 10^{-5,}$ in the SHEEP controls, were extracted.

Association between the selected SNPs and log IL8 values were calculated using a multivariate linear regression model (Beta (β) coefficients with corresponding 95% CI), under the assumption of an additive model of inheritance. Point estimates were adjusted by sex using STATA software. The same procedure was performed for each of the replication cohorts (Stage 2), independently.

When using case-controls studies, it is possible that the differences observed are a result of underlying genetic structure in the population and not a result of a disease associated locus (population stratification) (117). Thus, in the SHEEP, we further adjusted for principal components in order to correct for possible population stratification. Principal component is a statistical procedure that converts a set of observations of possibly correlated variables into a set of values of linearly uncorrelated values.

OR with 95% CI were calculated, through unconditional logistic models, to evaluate the association between rs12075 and MI in the SHEEP.

5.2.3 Study III

In the nested case-control design from the 60YO cohort, OR with 95% CI using conditional logistic regression model were calculated for the association of IL8 with CVE. The analyses were focused on comparisons of the values above the upper quartile with the lower quartile.

For the explorative analysis with CV mortality, serum IL8 median values were used as a cut-off for comparison, due to the relatively few amounts of events.

Potential confounding factors were defined as follows: diabetes at baseline was considered as having fasting plasma glucose ≥7.0 mmol/L and/or antidiabetic medication intake and/or having self-reported diabetes (108); Hypertension, defined as blood pressure ≥140/90 and/or antihypertensive drug therapy and/or self-reported hypertension (118); Hypercholesterolemia was defined as total cholesterol levels equal or above 6.45 mmol/L and/or treatment with

lipid lowering drugs. Smokers were defined as current smokers vs non-smokers or ex-smokers.

5.2.4 Study IV

To evaluate the association between sIL6R and sgp130 with the occurrence of MI, OR with 95% CI were calculated as estimates of IRR, using the same methodology as Study 1. Exposure for each biomarker was based on the cut-off value of the 75th and the 90th percentile of the distribution of the controls for the sIL6R and the sgp130, respectively. Only findings from the unmatched analysis were presented, as these were in close agreement with those from the matched analysis, yet allowed us to utilize all available information.

5.2.4.1 Biological interaction

The potential interaction between the two biomarkers exposures (sIL6R and sgp130) was analysed using departure from additivity of effects (biological interaction) according to Rothman and Greenland (119). When one of the exposures is preventive, such as sgp130 in our findings, the variable should be recoded such that the stratum with the lowest risk becomes the reference category for the co-exposure before calculating measures of interaction (120). Thus, the following model (*Table 2*) was constructed.

	Absence of exposure to high sIL6R	**Presence of exposure to high sIL6R**
Absence of exposure to low sgp30	Reference category	RR high sIL6R
Presence of exposure to low sgp130	RR low sgp130	RR combined exposure

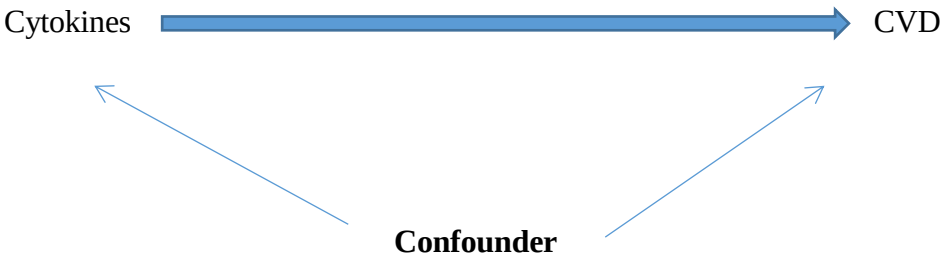
Table 2. *Model for the analysis of biological interaction. RR: relative risk*

To quantify the amount of interaction, the attributable proportion (AP) fraction and the synergy index (S) together with 95% CI were reported (121). In the interaction analysis sIL6R exposure was dichotomized into low ≤75th /high values >75th and sgp130 levels in serum were dichotomized according to the median percentile of the distribution. Because of low number of double exposures for certain stratum, we decided to use the median as cut-off for the sgp130. Analysis using as a cut-off the 90th percentile for the sgp130 distribution was also performed, yielding similar findings.

AP is the proportion of the incidence amongst individuals exposed to two interacting factors that are attributable to the interaction per se. AP greater than 0 indicates presence of interaction. An S index exceeding 1.0 indicates a synergistic effect, while an S index below 1.0 indicates antagonistic effect. It is the ratio between the combined effect and individual effects. OR and 95% CI were calculated using STATA and the measures of interaction were computed using Epinet sheet (122).

5.2.5 Selection of potential confounding factors

In all the studies, a similar approach was utilized when considering potential confounding on the associations under study. Single, as well as adjustments for combinations of covariates were performed, in order to evaluate their impact on the results obtained. Moreover, some covariates included in multivariate models in earlier studies were also considered when deciding what covariate to include in the model. The rationale behind the selection of the main potential confounding factors based on *á priori* knowledge in this thesis is explained below.



"A confounder must be associated with the disease (not with an effect of the disease) and with the exposure" (119)

Smoking: Nicotine has been reported to influence chemokine levels (123, 124). **Diabetes**: elevated concentrations of chemokines have been reported in diabetes type 2 (125, 126). **BMI**: elevated circulating cytokines has been reported among obese individuals (127). **Hypercholesterolemia**: Levels of cytokines and chemokines have been lowered in hypercholesteroleamic patients receiving statins. (128). **Hypertension**: cytokines have been implicated in the development and maintenance of hypertension (129-131). The above mentioned factors have been associated with an increased risk of CVD (2).

Additional adjustments for **job strain** (defined as low decision latitude but high psychosocial demands as determined by the Karasek-Theorell questionnaire), and **physical inactivity** (inactive leisure time) were also added as covariates, however not reported in the final models since its inclusion had little impact on the point estimates. Physical exercise has been reported

to reduce levels of IL8 after 12 weeks in patients with metabolic syndrome compared with baseline levels in a small group (132). Job strain has been associated to CHD risk after 12 years of follow up in a working population and a significant interaction effect with job strain was observed for IL8 levels in a study from the MONICA/KORA-Augsburg (133). It has been reported that cortisol may influence inflammatory responses via the hypothalamic pituitary adrenal axis through the induction of IL8 (134) and that epinephrine stimulates IL8 levels in samples containing platelets and stimulated with LPS (135).

CRP: CRP could be considered as an intermediate phenotype for IL8 and IL6 receptors, rather than a potential confounding variable. In Study 1, we had adjusted to observe if the findings indicated mediation.

Population Stratification: In Study II, different allele frequencies in a group of participants may be due to an ancestry difference, therefore population stratification may lead to a higher chance of finding spurious association and a lower chance of detecting true effects. Although the SHEEP population involves mainly citizens of Swedish origin, we initially include the first two PCs as covariates in the linear regression model in order to correct for population differences regarding SNPs that appear to be associated with disease. We did not see differences in point estimates, and adjustments without PC were reported.

6 RESULTS

6.1.1 Study I

Compared to lowest quartile of circulating IL8 in serum, study participants exposed to the highest quartile of IL8 were associated with a reduced occurrence of MI, particularly in women unadjusted OR 0.44 (95% CI 0.3-0.7). This association holds true after addition of potential confounders as covariates in the model.

The T allele at rs4073 was associated with a slight increased risk of MI under the additive and recessive model of inheritance, adjusted OR (95% CI) 1.2 (1.0-1.4) and 1.3 (1.0-1.7), respectively. In the stratified analysis, we saw an indication of effect measure modification where this association was only observed in men. Similarly, diplotypes carrying the T allele were associated with an increased risk of MI in men.

Variants in the genes encoding IL8 receptor were not associated with the risk of MI.

No statistically significant differences were observed among the serum levels of IL8 across the different genotype of each of the 5 SNPs for the *IL8* and *IL8R* genes studied in the SHEEP controls. It should be pointed out however that the lack of association with MI for these SNPs cannot be totally excluded, it may be that effects are small and that insufficient power did not permit detection of an association in the present study.

Compared to other biomarkers measured in the SHEEP, we observed a statistically significant difference in IL8 levels measured in cases at different time points after the disease onset, particularly in women.

6.1.2 Study II

From the 121,445 SNPs that passed quality control, five SNPs (rs12075A/G mapping at chromosome (Chr) 1, rs13179413C/T at Chr5, rs6907989T/A at Chr6, rs9352745A/C at Chr6 and rs1779553T/C at Chr14) were associated with log transformed IL8 in serum at a p-value $< 1x10^{-5}$ in the discovery stage, with the strongest association SNP (rs12075 A/G, unadjusted p value $1.6 x10^{-6}$) mapping at the *DARC* gene.

These five SNPs were carried forward to *in silico* replication in the PIVUS, MDC-CC and SCARF studies. Replication was defined as a p-values <0.05 for the association evaluated in the independent populations.

The MAF for each SNP were rather similar across the different populations. Consistent with observations in the SHEEP, the association observed between SNP rs12075 with log serum

IL8 levels was replicated in the PIVUS study (p=0.0006), where a 10.4% reduction in IL8 was observed in the presence of the G allele. These findings were not replicated in the MDCC-CC and SCARF studies.

Similarly, the association observed at the other 4 SNPs could not be replicated in serum nor plasma in the different studies.

Homozygous carriers of the G allele at rs12075 SNP had lower serum median (IQR) IL8 levels (AA =17.1 (9.9-24.6) pg/mL; AG =13.5 (8.9-20.8) pg/mL; GG=11.2 (7.4-16.9) pg/mL) in the SHEEP and in the PIVUS (AA =7.2 (5.0-10.7) pg/mL; AG =6.3 (4.5-9.0) pg/mL; GG=6.0 (4.0-8.3) pg/mL) studies. The decreasing trend of IL8 in plasma was similar across the SCARF and the MDC-CC studies, although there was no statistically significant difference.

Further, IL8 was measured in plasma in a random subset of the SHEEP individuals where samples were available. In this group, no association was observed between the 5 SNPS with log IL8.

We did not observed an association between the SNP rs12075 and the risk of MI in the SHEEP using the additive OR 1.0 (95% CI 0.9-1.1), dominant OR 1.0 (95% CI 0.9-1.1), or recessive OR 1.1 (95% CI 0.9-1.1) model of inheritance, after adjusting for the matching variables of the study.

6.1.3 Study III

In the crude analysis, exposure to high levels of IL8 (highest quartile) were not associated with increased risk of CVD in both women and men, unadjusted OR (95% CI) 0.8 (0.4-1.5) and 1.0 (0.7-1.6) respectively, compared to the lowest quartile. Further adjustments for hypertension, diabetes, hypercholesterolemia, BMI and current smoking did not lead to major changes in the point estimates.

An interesting finding was indication of an association between high serum levels of IL8 and CV mortality, particularly in men, when using the 50^{th} percentile as a cut-off (confidence intervals, though, were wide and overlapping one): OR of 2.4; 95% CI (0.8-7.1). However, the power to detect such association was rather low, especially in women.

6.1.4 Study IV

Exposure to high serum sIL6R was associated with increased occurrence of MI, even though the associations were weakened after adjusting for possible confounding factors. Using as

cut-off limit the 75th percentile value in the control group, the OR was 1.4 (95% CI 1.1-1.8) after adjustments for age, residential area, hypertension, diabetes, hypercholesterolemia, BMI and smoking. Even higher point estimates were observed when using the 90th percentile value in the control group as the cut-off limit OR: 1.7 (95% CI 1.2-2.3). Conditional logistic regression models were also evaluated, yielding similar point estimates as the unconditional models: OR was 1.5 (95% CI 1.2-1.9) for the crude model and in the adjusted model OR was 1.4 (95% CI 1.0-1.8).

For levels of sgp130, there was no association with MI when using, as cut-off limit, the 75th and the 90th percentile value in the control group in the crude analysis. However, after adjustments, exposure to very high sgp130 (>90th percentile) was associated with a reduced occurrence for MI, OR 0.68 (95% CI 0.5-0.9). Particularly, adjustments for diabetes and hypertension led to lower ORs and CIs suggesting their influence as confounding factors.

There was an indication of a possible biological interaction for the co-exposure to sIL6R and sgp130. High sIL6R levels (>75th percentile) and low sgp130 (below the median) yielded S index point estimates of 1.7 (95% CI 0.5-6.1) and an AP fraction of 0.19 (95% CI -0.2 – 0.5).

7 DISCUSSION

7.1.1 Circulating IL8 and cardiovascular events

Study I indicates that elevated levels of IL8 were associated with a decreased occurrence of first time MI in a population aged 45-70 years old living in Stockholm. At the same time, high levels of IL8 were not associated with increased risk of CVE in a cohort of 60 years old men and women from the Stockholm area (Study III).

Findings from population-based observational studies investigating the association of IL8 with CVD risk appear inconsistent across countries, materials and methodological approaches (72, 73). In addition, published studies are few, likely due to publication bias since this has been pointed out as a major challenge in biomarker research (136, 137). Publication bias arises when published findings differs from the results of *all* the unpublished research that has been done in an area.

When comparing the present results to previous studies, several aspects including study design, endpoints, length of follow-up, as well as potential confounding factors must be taken into consideration.

7.1.1.1 Study design

Sampling in the SHEEP was performed at least 3 months after MI, at which time, metabolic stability should be regained (138, 139).

To interpret our findings in the context of our population, we have characterized the trend of IL8 in cases sampled at different times, comparing levels of median IL8 analysed according to sampling time-frame. Whereas other biomarkers previously reported in the SHEEP such as IL6, CRP, TNF-α seemed to be rather stable in cases across sampling times, the pattern of IL8 appears to differ according to sampling date, particularly in woman. This analysis, though, needs to be interpreted with caution, since these are not case repetitive measurements and the inter-individual variability was not taken into account. In addition, it might well be that those with a more "severe" form of disease attended the clinical investigations earlier or later as compared to the rest of cases, although this cannot be determined.

A second way in which the event itself might have influenced the exposure levels is with regards to medication post MI. Therapy prescribed for secondary prevention, in particular with acetyl salicylic acid (ASA), might influence the levels of IL8, and therefore potentially confound the association we observed in the SHEEP. Indeed, at a dose -response, ASA has been shown to down-regulate IL8 at the mRNA and at the protein levels in TNF-α stimulated

HUVEC (140). The lower levels of circulating IL8 observed in cases as compared to controls might be in relation to the large proportion of cases with ASA. However, at low doses, aspirin exerts mostly antithrombotic rather than anti-inflammatory effects (141). Little is known with regards to the role of circulating IL8 in arterial thrombosis and the effect of thromboxane inhibition by aspirin on circulating IL8. In this context, other investigators have found increased expression of IL8R in platelets obtained from patients with inflammatory bowel disease (142). At the same time, IL8 has been shown to inhibit megakaryocytopoiesis suggesting its involvement in platelet modulation and activation (143).

In light of the unexpected findings with the SHEEP (Study I), we have sought to further adjust for medication therapy. Inclusion of these covariates had little impact on the results.

In conclusion, reverse causality as an explanation to findings from the SHEEP cannot be entirely ruled out. Interestingly, the association of IL8 with CVD in the 60YO, where the exposure was collected before disease onset, showed point estimates rather consistent with the SHEEP findings particularly in women, although with wide confidence intervals.

7.1.1.2 Differences in Cardiovascular Endpoints

For the purpose of increasing power in a single endpoint, often in epidemiological studies, fatal and non-fatal CVE are combined. Unlike other cohort design studies, the results from the SHEEP study are restricted to non-fatal MI. The *EPIC Norfolk* study had a composite outcome of non-fatal and fatal CAD, defined as participants who had a hospital admission and/or died with CAD as underlying cause using the 9th ICD codes 410 to 414. These codes identified acute MI, angina pectoris and other forms of CHD. On the other hand, the *MONICA/KORA* Augsburg study defined the outcome as incident fatal, non-fatal MI and SCD before age 75. The cohort of 60YO defines a composite outcome of fatal and non-fatal MI, hospitalization due to angina pectoris as well as fatal and non-fatal strokes.

Although all these events may share common aetiology, it is still unknown if the difference among fatal and non-fatal outcomes is only in severity or whether there are subtle differences in the underlying mechanisms. High levels of IL8 were associated with a slight increased risk of all-cause mortality in a Swedish cohort of elderly women from the PIVUS study (144). Furthermore, in a secondary analysis from the *CORONA* trial, IL8 was associated with increased risk of all-cause mortality (75). As a result, survival bias might explain at least part of the discrepancy in findings from the SHEEP study, as compared to earlier studies. We observed an indication of an association of IL8 with CV mortality in the 60YO cohort and it is tempting to speculate that CV mortality might have been a driving force in the associations

previously reported. Nonetheless, our study comprises few CV deaths and further studies are needed to confirm this hypothesis.

At the same time, the 60YO cohort includes, as an outcome, ischemic stroke, a disease that shares common features with the pathophysiology of MI (145). IL8 levels has been reported to be elevated in atrial fibrillation, a condition that predisposes to stroke and thrombo-embolisms (146). Yet, to the best of our knowledge, the association of IL8 with angina pectoris and stroke is unknown. Noteworthy, in the 60YO cohort, IL8 at baseline did not differ significantly among MI, angina pectoris and ischemic stroke, thus, the use of a different composite outcome might not fully explain the differences observed. On the contrary, IL8 at baseline from fatal cases was higher as compared to the rest of the study population.

Inflammatory biomarkers may not be causally related to CV death. Instead, our results could support the hypothesis that inflammatory biomarkers, at high concentrations, may reflect subclinical disease or "biological ageing (147) . Previous epidemiological studies in the elderly have underscored the notion that risk estimation in elderly people may warrant an altogether different approach than in young and middle aged subjects, since inflammation (IL6, CRP) has been found to be associated with a graded risk of all-cause and CV mortality in elderly men (148). In addition, IL6, CRP and to a lesser extent, fibrinogen are more closely associated with the risk of fatal MI or stroke than to non-fatal CVE in the elderly (149). The reason why the association between IL8 and CV mortality was predominantly observed in men after adjusting for traditional cardiovascular risk factors in Study III is unclear.

Overall, the overriding influence of age in a "cohort of survivors" may have underestimated any of the associations, suggesting that it may be differences in risk factor patterns, perhaps with regard to inflammatory biomarkers between fatal and non-fatal events. Consequently, it may be necessary to dissect association of IL8 with fatal and non-fatal events.

7.1.1.3 Differences in baseline characteristics

It is plausible that IL8 measurements at baseline may mirror a chronic inflammatory state, such as cancer or other chronic inflammatory diseases not yet diagnosed at the time of enrolment. It is possible that improved survival in CVDs in developed countries (150) is accompanied by an increased risk of cancer incidence and mortality.

In Study 1, the decreased occurrence of MI with high IL8 levels was observed particularly in women. Speculatively, some of the potential mechanisms that may explain these findings are the decreased concentration of estradiol accompanied in the transition from perimenopausal

to postmenopausal stage. IL8 has been reported to increase after menopause and has been correlated with symptoms such as hot flashes (151, 152). However, our findings were not explained by the use of HRT. Approximately 43% of women in the 60 years old cohort were under HRT and adjustments for this variable did not change the point estimate either.

IL8 release has been shown to be higher in human visceral adipose tissue as compared to subcutaneous adipose tissue (153). Women tend to have a higher percentage of subcutaneous fat than men, despite comparable amounts of visceral adipose tissue (154). However, aging has been reported to increase the visceral adipose tissue with weight gain in women (155). In study 1, we have further adjusted for waist circumference and the point estimates did not change substantially.

7.1.1.4 Experimental studies

Experimental studies in animals have been hampered by the fact that, to date, a homologue of human IL8 has not been identified in rodents, (156) although human IL8 is partially functional on the corresponding IL8R in mouse (157). Thus, translation derived from in vitro studies in clinical setting remains to be elucidated.

Experimental evidence has also indicated that IL8 prompts tissue protective effects during ischemia reperfusion injury (158), wound healing (159) and tissue remodelling (62).

7.1.1.4.1 IL8 isoforms

IL8 isoforms seem to display heterogeneity with respect to chemotaxis of inflammatory cells. Studies in rabbits indicated that in vitro, the $[Ser\text{-}IL8]_{72}$ isoform is approximately 10 fold more potent than the $[Ala\text{-}IL8]_{77}$ in inhibiting adhesion of neutrophils to IL1 activated cultured ECs (67, 70). Nevertheless, in vivo, these observations were not consistent, since intradermal administration of both isoforms produced equipotent results (67). Another study in a model of intradermal inflammation in rabbit, reported that intravenous administration of the IL8-77 isoform inhibited progressively polymorphonuclear (PMN) accumulation (160), a similar finding for the 72 isoform in a dose-response . However, when injected into the dermis, both isoforms showed PMN accumulation with comparable activity. In neonates, while inhibition of total IL8 decreased chemotaxis, the inhibition of IL8 77 has no effect (71). Thrombin stimulates production of IL8 (161, 162) and, together with plasmin, efficiently converts $[Ala\text{-}IL8]_{77}$ to $[Ser\text{-}IL8]_{72}$ (70, 163). Antithrombin III inhibits the conversion of $[Ala\text{-}IL8]_{77}$ (160). Dysfunctional endothelium may exhibit procoagulant properties and can potentially generate thrombin (160) , and therefore the conversion of $[Ala\text{-}IL8]_{77}$ to the more

potent isoform may occur at the interface of the vessel wall and blood in sites of inflammation.

Altogether, it could be conceivable that a shift of isoforms might partly explain the results obtained in epidemiological studies. Thus, it may be of interest to estimate the proportion of circulating isoforms in population based studies and during acute inflammatory conditions, as well as to explore if there are isoform differences in terms of affinity to both receptors. The heterogeneity in epidemiological findings reported so far for IL8 and risk of CVD raises the possibility that it could be related to different proportions of circulating isoforms.

In a broader perspective, all these observations may prompt the hypothesis that the pleiotropy of IL8 activities exhibited depends on whether this chemokine is active extravascularly or intravascularly (160). It might well be that the proinflammatory properties of IL8 are related to intraplaque and intralesion activity, while the regulation (anti-inflammatory IL8) is driven by the circulation. Therefore, circulating IL8 might not be a proxy for in situ inflammation, and the lack of associations would not preclude the local chemokine effect on coronary arteries and atheromas.

7.1.1.4.2 IgG and DARC

Furthermore, studies conducted during the 90´s have reported a neutralizing antibody (IgG) that binds to IL8 in serum. This complex (IL8-IgG) is not able to bind to IL8 receptors in neutrophils and possibly to human erythrocytes in vitro (164, 165).

Although the functional significance of these antibodies are unknown, it is plausible that, along with erythrocyte sequestration by *DARC*, stored IL8 limits the biological effect of IL8 that gains access to the circulation. These mechanisms may facilitate the elimination of residual IL8 not internalized by erythrocytes. Regardless of their physiologic role, these antibodies are potentially confounding variables in the quantification of IL8 in biological fluids as they may compete in immunoassays or bioassays used to detect IL8.

7.1.2 Genetics variants of IL8 and risk of MI

Genetic association studies of a particular genetic variant are challenging. Furthermore, the effect of alleles at a given gene may depend on the combination of genes at other loci not being studied, and therefore alter the strength of the association (76).

Since there is no phenotype data to hint at an indication to use one inheritance model over another, the multiple models approach was used to investigate the genetic variants (166).

In study I, a candidate gene (i.e. hypothesis driven) approach was used to identify SNPs potentially associated with the regulation of IL8 as well as with the development of MI. We have observed that a promoter genetic variant at the IL8 gene was associated with a slightly increased risk of MI under an additive and recessive model of inheritance. Consistently with these results, the same variant was associated with an increased risk of ACS using a co-dominant model in a Chinese Han population (85). On the contrary, in a Caucasian population with CAD, the common $AA_{251}TT_{781}$ genotype of the *IL8* gene was associated with a reduce risk of ACS (84). In the latter study, the reference category was those with the most common genotype. However, most studies were small in size, and the majority of the reported findings are unconfirmed. The same genetic variations have been involved in the susceptibility to a large number of inflammatory diseases and allele frequencies show diversity in the different ethnic groups (167-169), an observation that has been previously highlighted by others (170, 171). Of importance in the interpretations of these findings is that a considerable amount of missing genotypes was observed for the SHEEP. The missing genotypes were randomly distributed in cases and controls. However, the MAF of the SNP rs4073/-251A>T in the SHEEP was comparable to the frequency reported in other European populations (172, 173), but not to that reported in the Hapmap and the 1000 genome projects.

Haplotype analysis uses additional information regarding the linkage between genetic variants mapping at the same gene. For example, even though rs4073 is situated at the promoter region, it may, through linkage with other polymorphisms, located within other regulatory or coding regions, affect gene transcription or function resulting in interindividual variation in levels of cytokine production/activity. Results of haplotype frequency estimation for two-SNP combination at the *IL8* gene (rs4073A/T-rs2227306 C/T) suggested that diplotypes carrying the T allele at the two SNPs were associated with an increased risk of MI, particularly in men.

In study I, IL8 levels did not differ significantly across genotypes in the control group at each of the *IL8* gene and *IL8R* gene. Published studies in this respect are scarce. An earlier study on biopsy specimen from mucosal IL8 levels of the gastric body have reported that, as compared to the T allele, carriers of the A allele of the rs4073 had significantly higher IL8 protein (174), and similarly, experimental data suggested that the A allele is associated with increased IL8 production (172). Carriers of the TT genotype where presumed lower IL8 production is observed had a poorer survival for follicular lymphoma (175). All these observations underscore the complexity of disrupted pathways in IL8. However, cautious interpretation of these findings is necessary, due to lack of replication.

In light of these latter observations, Study II has sought to investigate the genetic determinants of IL8 using SNPs contained in the Cardiometabochip. In controls belonging to the SHEEP study, the SNP rs12075 coding *DARC* gene was associated with log transformed values for IL8. Lower values of IL8 were observed in the presence of the G allele. While the association was not at a GWAS level of significance, the findings were replicated in independent sample sets.

DARC, recently named as an atypical chemokine receptor (176), is expressed in erythrocytes, post-capillary ECs and Purkinje cells. DARC shares two interesting features: first, it lacks the DRY motif that is required for signal transduction, acting as a "silent decoy receptor" and it only binds angiogenic, but not angiostatic, chemokines. Therefore, DARC may act as a key regulator of IL8 highly dependent on its localization: in erythrocytes, this protein serves as a reservoir for IL8 whereas in ECs it may facilitate IL8 translocation through the vessel wall (177, 178). IL8 bound to erythrocytes is not bioactive and this mechanism may play an important role in controlling the inflammatory response driven by this chemokine (179). In the present context, such binding may have had an appreciable impact on the amount of circulating IL8 detected and might hamper the interpretation of measurable IL8.

The previous findings evoke testable hypotheses regarding the role of *DARC* genetic variant in CVD by damping inflammation (180). Thus, we have evaluated rs12075 SNP in relation to MI in the SHEEP, where no association between *DARC* and MI was observed. Although this genetic finding has, as yet, not been replicated in other studies, previous genetic consortia have not reported an association between this particular SNP and the risk of CVE. However, it is uncertain if one may find a lack of association considering atherosclerosis as an outcome. IL8 has been found in atherosclerotic plaques (54) and it is further conceivable that those carriers of the minor allele might have a lower risk of developing atherosclerosis.

An intriguing finding was the fact that the association of rs12075 with IL8 could only be replicated in the cohort where IL8 was measured in serum. We did not investigate in depth the molecular mechanisms to clarify these genetic differences that were observed. We did, however, measure IL8 in plasma samples in a subset of the SHEEP controls with available measurements of IL8 in serum, and the findings could not be replicated. Serum is plasma without the clotting factors. Neither plasma nor serum contains red or white blood cells or platelets. A comparison of serum and plasma IL8 has shown much higher levels in the former possibly, because like other chemokines, IL8 might be released on platelet activation during clotting (181, 182).

Currently, there is no consensus of which biological fluid provides more accurate assessment or standardized procedures for IL8. In line with these results, it has been suggested that chemokine release upon platelet activation during the clotting process makes serum measurements artefactually high, (183), making plasma a better choice. On the other hand, low levels of chemokines lie at the current limit of detection of many available assays, thus serum assessments may give greater sensitivity. It could be conceivable that IL8 serum levels, corrected for the blood platelet count, might be the best way to assess IL8 circulating in blood (184).

7.1.3 Circulating sIL6R and sgp130 and risk of MI

7.1.3.1 sIL6R

Considering that many cytokines orchestrate a specific biological response, it seems reasonable in epidemiological studies to investigate multiple cytokine within a particular family in order to enhance our understanding of the inflammatory response (185). Therefore, studies of the two other constituents of the IL6 *trans-signalling* (sIL6R and sgp130) may give more precise information about the activation of the IL6 pathway (186).

We have observed an increased susceptibility to MI in individuals exposed to high sIL6R in the SHEEP. No dramatic influence by any specific covariate was observed. However, a number of covariates had moderate influence on results, but often only in combination with other covariates.

In agreement with these results, elevated levels of sIL6R have been observed in patients with MI (103), CHD (187), in elderly men with metabolic syndrome, endothelial dysfunction, arterial stiffness (188) and in other inflammatory diseases such as anti-citrullinated cyclic peptide antibody in rheumatoid arthritis (189). In addition, experimental studies have implicated IL6 *trans-signalling* in the pathogenesis of Chron disease (190), pulmonary fibrosis (191) and colorectal cancer (192). However, evidence from population-based studies is lacking.

Recently, Rose-John and colleagues has widely discussed a buffer system underlying the *trans-signalling*, where sIL6R and sgp130 form a buffer for IL6 in the circulation (93). Under this premise, sIL6R will bind with an affinity of around 1nM to IL6 and once the complex is formed, it will bind to sgp130 with 100 times higher affinity (10 pM) to neutralize the complex (193). It could be speculated that in the SHEEP, the sIL6R measurements mirrors

the sIL6R/IL6 complexes that are activating the *trans-signalling*. Another interpretation, might well be, that cases would have higher "free" sIL6R to bind IL6 to activate the *trans-signalling.* The measurements in the present study, however, cannot distinguish the complex sIL6R/IL6 and sIL6R.

7.1.3.2 Sgp-130

Different cut-off values were used for evaluating the association of these biomarkers with MI. When using different cut-off values based on the 75th and 90th percentile, no association was observed in the crude models. Only very high levels (>90th) of sgp130 were associated with a reduced occurrence for MI in the adjusted model, suggesting the influence of potential confounders in this association. When adding the covariates to the regression model one by one, diabetes was observed to have the largest impact on the point estimates. Associations have been reported between plasma sgp130 levels and metabolic syndrome in an elderly community, possibly mediated by insulin resistance (186). Furthermore, serum sgp130 was inversely associated with insulin sensitivity in women with polycystic ovarian syndrome (194), suggesting a complex interplay of the *trans-signalling* in different metabolic conditions.

As for the sIL6R, the sgp130 measurements were not able to determine the "free" circulating levels, from the sIL6R/sgp130/IL6. One may speculate in both cases, that inhibition of the *trans-signalling* pathway may be associated with a reduced susceptibility to MI.

7.1.3.3 Biological interaction

Knowledge of potential interaction between two biomarkers would certainly contribute to direct, experimentally orientated research towards important biological mechanisms. The measures of interaction presented in Study IV, possibly suggest the presence of biological interaction among the two co-exposures. It must however be noted that evidence of synergism or antagonism with this analysis requires larger study materials than the present to obtain more stable estimates.

It has been discussed that blocking the IL6 *trans-signalling*, while maintaining the physiological classic signalling, holds the promise of developing selective *trans-signalling* inhibitors. In fact, a recent study demonstrated that inhibition of the *trans-signalling* of IL6 through the administration of sgp130 significantly reduces the development and progression of atherosclerotic plaques in mice, by neutralizing IL-6/sIL6R complexes without affecting classic signalling, i.e., acute phase response and cholesterol levels (101). Recombinant

proteins that mimic the property of sgp130 have been developed and are currently in clinical trials for the treatment of inflammatory diseases (195).

As outlined earlier, a SNP causing an Asp328Ala aminoacid change in the membrane bound IL6R has been identified. Remarkably, carriers of the minor allele of the Ala358 present lower levels of CRP (51, 196) but at the same time elevated levels of IL6, sIL6R (96, 97, 197), and yet are less susceptible to type I diabetes and CHD (51, 96-98). In this context, increased levels of sIL6R appear to have an anti-inflammatory impact. More recently, IL6R haplotypes has been reported to regulate serum levels of CRP, IL8, sIL6R and fibrinogen (198), suggesting a complex interplay in the regulation of inflammation.

8 METHODOLOGICAL CONSIDERATIONS

Strengths

The major strength of case-controls studies such as the SHEEP is the large number of subjects with available extended exposures information and biological samples. Furthermore, this design offers an alternative that is much more efficient compared to the corresponding cohort.

The high participation proportion of those invited to participate in the cohort of 60YO allows this study to be representative of 60 years old women and men from the Stockholm area with complete follow-up. The combination of a large study sample and high costs for new technologies makes it unfeasible to measure new markers on an entire cohort. Thus, an additional strength of the nested case-control lies in reducing the cost of exposure assessment in the prospective cohort.

These studies were carefully designed and controls were randomly sampled from individuals at risk, at the times when cases were identified. With this approach (incidence density sampling), controls mirrors the exposure frequencies in the study base that generated the cases. By matching procedures, the aim was to prepare for a more efficient stratified analysis. Increased efficiency is achieved if there is a strong association between the matching factor and the disease under study. However, matching implies that is not possible to investigate the associations between the matching factors and the disease of interest.

Reverse causality

Temporality (exposure precedes the outcome) is an issue to consider in a case-control study, where sampling was performed at least 3 months after the MI event. In Studies I and IV, one cannot be certain that the serum concentration of biomarkers observed truly reflected the exposures before disease onset. It is therefore not possible to draw conclusions on causal relationships between the exposure and the disease. However, in young post MI patients, Hamsten and co-authors have shown that some biomarkers regain metabolic stability 3 months after the disease onset (138, 139). This is supported by the level of other biomarkers reported in the SHEEP (IL6, TNF alfa, fibrinogen), which are comparable to baseline levels of prospective studies (87, 199, 200). This potential source of error, may lead to an over or underestimation of the OR which is not possible to correct in data analysis. However, the genotype variables in Study I are unlikely to be affected by this potential source of error.

It is likely that cases follow preventive measures recommended by the physician after the index event. It is therefore reasonable to believe that changes in lifestyle pattern e.g., physical activity, diet and smoking cessation post MI may influence levels of biomarkers under study. On the other hand, a recent systematic review reported insufficient evidence for the effect of physical activity on IL8 (201) . Diet components such as Vitamin D, has been shown to decrease IL8 secretion from preadipocytes (202). Such changes might have yielded underestimated results.

The proportion of hypertension was lower in cases as compared to controls. Blood pressure commonly falls after an MI event due to damage in the myocardium and might, to some extent cause an underestimation in patients. Other medication such as statins, and beta-blockers, mainly prescribed in cases, could possibly have an influence on the levels of IL8 (128). However very few individuals were receiving statins, since the first large scale cholesterol lowering trial suing statins was yet to be published by the time the SHEEP data collection was performed (203). In human carotid atherosclerotic plaques, lower neutrophils were observed among individuals using beta-blockers as compared to non-users, and at the same time high neutrophil numbers were associated with high levels of IL8 (204).

Thus it is important to consider the potential inherent bias linked to the retrospective post-event data collection.

Measurement aspects

In the SHEEP and the cohort of 60YO, samples contained at bio-banks were frozen over a long period of time before analysis. The number of epidemiological studies involving prolonged blood storage is overflowing. Such investigations using different biomarkers stored at biobanks have yielded comparable associations with CVD risk (at least as strong as in former studies), which strengthen the case against underestimation due to sample degradation (205). Yet, to date, there is no available epidemiological study aiming to investigate this issue. It is likely that in this scenario, the misclassification would occur, with more or less, equal frequency regardless of the disease status. Non-differential misclassification of a dichotomous exposure biases toward the null (Studies IV). However, if the exposure is not dichotomous, then non-differential misclassification may bias the estimate either toward the null or away from it, depending on the categories into which participants are misclassified (Studies I, II and III).

All the biomarkers of the present study were measured at a single time point. In Study III, the possible fluctuations in biomarkers over time may yield biased estimates of the true

association ("Regression dilution") (206). Such bias is likely to be non-differential leading to attenuation of the true associations.

In addition, in the SHEEP study n=121 samples and n= 3 samples were thawed once and twice respectively. In order to minimize the potential source of error, time of defrosting was handled similarly among cases and controls Adjustments for number of thawing occasions did not alter our findings and it was randomly distributed among cases and controls.

Non-participation

In epidemiological studies, non-participation may result in selection bias. Selection bias arises when participants selected are different from those not participating.

In general, the high participation proportions in the SHEEP and the cohort of 60YO studies make them less vulnerable for selection bias. Still, it might be possible that non-respondents differ from respondents with regard to their pattern of exposure. In the SHEEP, for instance, controls had a lower participation proportion as compared to cases. If such differences are present, differential non-participation may yield underestimated results.

One may argue that not all samples from the original SHEEP design were analysed. However the lack of enough serum samples with regards to each biomarker was similarly, and randomly, distributed among cases and controls, so it is unlikely that a selection bias might have an impact in our estimates. Nevertheless, in the genetic analysis for the promoter SNP rs4073, a considerable amount of study participants were missed, therefore we cannot exclude the possibility that these results might be due to chance. Further replication in independent cohorts is warranted.

In order to address the question of a potential selection bias introduced by the proportion of individuals excluded due to missing samples in study IV, baseline characteristics from these study participants were re-analysed. Compared to the entire SHEEP population, those participants who were excluded were slightly younger. Hypertension was slightly more prevalent in controls as compared to cases, an observation consistent with the SHEEP population. Sex distribution, BMI, diabetes, hypercholesterolemia, physical inactivity and smoking presented similar prevalence.

The practically complete to follow-up (99.8%) of the 60YO through linkages to various population-based registers minimize the possibility that our results were subjected to differential loss to follow-up.

Misclassification of Exposure

Measurements processes were conducted blinded with regard to case-control status, in order to minimize the "observer expectation bias" in the nested case-control of the 60YO. In the majority of occasions, the SHEEP samples were placed in a similar proportion with regards to case-control distribution in each plate, and were read in a "blinded" way by the machine. Systematic genetic differential misclassification of exposure is unlikely.

Recall bias

Cases may recall exposures differently at the time of filling in the questionnaire compared to controls. This is an inherent limitation of case-control study designs, such as in the SHEEP. The exposures under study in this thesis were not collected through questionnaire; however, covariates such as smoking were reported through questionnaires. Any potential misclassification in respect of confounders should also be taken into consideration.

Survival bias

The inclusion of only non-fatal MI events may not truly generalize our results on fatality cases (n=603) in the SHEEP study. However, due to the study design it was impossible to perform sampling on fatal events. Data regarding biomarkers of fatal cases from large epidemiological studies are quite rare. This raises the question whether the exposures studied in this thesis are different (lower) from those in fatal outcomes. One may therefore keep in mind that the ORs in Studies I, II and IV may be an underestimation of the impact of these biomarkers on the occurrence of MI.

Misclassification of Disease

From the period when the studies included in this thesis were conducted up to now, more sensitive and specific diagnostic methods for MI have developed. Although, these new diagnostic criteria will be more sensitive for smaller MIs, clinically silent MIs are still frequently in advanced CHD. Thus, it is important to consider that MI cases considered in these studies refer to only those clinically diagnosed, which may only reflect a subset of all MIs. Such silent infarctions, if present, were not excluded from the study base, since they could not be ascertained amongst the participants of the present studies.

Residual and unmeasured confounding

When studying inflammatory biomarkers, it is reasonable that they might also mirror inflammatory conditions that are not in the scope of the study. In fact, cytokines have been

associated with several cancers and other inmunoinflammatory diseases (207, 208). We did not adjust for these inflammatory conditions since, unfortunately, we did not have specific information. As well, environmental factors with potential influence on the biomarkers in this study were not considered in this thesis. The presence of unknown confounders cannot be excluded.

9 CONCLUSIONS AND FUTURE PERSPECTIVE

The current findings of this thesis may have implications for understanding disease mechanisms and for further research strategies.

In a population of 45-70 years old, elevated levels of IL8 in serum was associated with a reduced occurrence of non-fatal MI. Furthermore, no association was observed with a combined endpoint of fatal and non-fatal CVE in a 60 years old population; however there was an indication of a possible association between IL8 with risk of CV mortality.

This finding may suggest that IL8 might serve as a marker of disease severity in older adults, but not to the same extent in younger population, free of prevalent MI. In this context, the question arises as to whether levels of IL8 reflect inflammation in the atherosclerotic plaque itself, or inflammation at another site, with secondary effects on the vascular wall. Molecular epidemiological studies designed to unravel the potential relationship between IL8 with CV and cancer mortality, as well as non-fatal MI should be of interest. In particular, further assessment of IL8 isoforms and anti-IL8 will be necessary to elucidate the behaviour of this chemokine in the spectrum of CVD.

IL8 in serum did not differ among genetic variants at the *IL8* and *IL8R* gene. One SNP at the promoter region of the *IL8* gene was associated with a slightly increased risk of MI. However, it is important to realize that these results must be repeated in other epidemiological materials before robust conclusions are drawn. A genetic variant coding the *DARC* gene was associated with IL8 in serum and, surprisingly, not with IL8 in plasma. Although initially confusing, these observations provide additional insight into the physiological and pathophysiological roles of this chemokine.

Taken together, these results suggest that interpretation of circulating levels of IL8 in epidemiological studies should take into consideration the amount of IL8 internalized by the *DARC*, genetic predisposition, baseline characteristics of the population under study, time at which the blood sample is taken and whether the measurements are performed in serum or plasma.

Elevation of the sIL6R and sgp130 had opposing associations with the occurrence of MI, and sgp130 was an effect modifier of the association between sIL6R with occurrence of MI. Furthermore, we observed an indication of a possible synergism between high sIL6R and low sgp130 with risk of MI. However, the lack of power to detect additive interaction requires further, larger studies. Future studies that will be of interest will be those aiming to replicate

these findings as well as to investigate whether the potential benefit observed with higher levels of sgp130 may be more substantial in a particular group of individuals.

There may well be additional mechanisms at play which are beyond the scope of this thesis. Still, a number of crucial questions remain. First, can circulating levels of cytokines reflect the vascular state reliably enough to allow prediction of CVE? Indeed, to avoid misleadingly optimistic results, future studies are required to better assess the variability of biomarkers across time, as well as the impact of blood storage. Secondly, is the role played by IL8, sIL6R and sgp130 during the early and later stages of atherosclerosis similar? Thirdly, are the potential protective or deleterious effects of these biomarkers directly dependent upon their concentration? Answers to these questions may help elucidate the delicate risk-benefit balance and winding path involved in anti-inflammatory interventions.

10 SUMMARY IN SWEDISH

Syftet med den här avhandlingen var att undersöka sambandet mellan inflammationsmarkörer och ischemisk hjärt-kärlsjukdom .

Specifika syften var att studera sambandet mellan nivåer av IL8, såväl som genetiska varianter av *IL8* och *IL8* receptorer och hjärtinfarkt. Dessutom var syftet att undersöka om dessa genetiska varianter reglerar cirkulerande IL8-nivåer (Studie I); identifiera nya genetiska varianter associerade med cirkulerande IL8-nivåer (Studie II); studera sambandet mellan IL8 nivåer och risken för kardiovaskulära händelser (Studie III) och slutligen att undersöka sambandet mellan nivåer av lösliga IL6 receptorer (sIL6R och sgp130) och förekomsten av hjärtinfarkt.

Material och metod: Studie I, II och IV baserades på Stockholm Heart Epidemiology Program (SHEEP), en populationsbaserade fall-kontrollstudie. Fallen utgjordes av incidenta fall av hjärtinfarkt under perioden 1992 till 1994 som inträffade på 10 akutsjukhus i Stockholms län. Kontrollerna var slumpmässigt utvalda från studiebasen via density sampling och matchade för ålder, kön och sjukhusens upptagningsområden. I analyserna användes enbart fall av hjärtinfarkt utan dödlig utgång (n=1243) och matchade kontroller (n=1561) med tillgänglig data om biomarkörer. Exponeringsinformation insamlades via enkäter, medicinska register, blodprov och antropometriska mätningar. Studie III är en ”nested” fall-kontrollstudie baserad på en kohort av 60-åriga män och kvinnor (60YO), som genomfördes mellan 1997 och 1999 i Stockholms län. Antal personer i studien var 4232. Fram till 31 december 2012 har 491 hjärt-kärlhändelser registrerats. För varje fall valdes könsmatchade kontroller (n=981) med liknande uppföljningstid. De var fortfarande under risk att insjukna (ej blivit fall under uppföljningsperioden), och ingick i kohorten 60 dagar före eller efter att den person som blivit fall inkluderats i kohorten.

Resultat och slutsatser. I SHEEP var låga cirkulerande IL8-nivåer kopplade till förekomst av icke dödlig hjärtinfarkt (Studie I). I 60YO fanns inga samband mellan IL8 och risken för kardiovaskulära händelser (dödlig och icke dödlig hjärtinfarkt, dödlig och icke dödlig stroke och slutenvårdsbehandlad angina pectoris) (Studie III). IL8’s mediannivåer varierade inte med genetiska varianter av *IL8* och *IL8* receptorer. En SNP rs12075 A/G, Asp42Gly, som kodar för Duffy Antigen Receptor for Chemokines (DARC), visade på skillnader i nivåer beträffande IL8 i serum men inte i plasma. Lägre IL8-nivåer observerades hos personer med ’’2 kopior’’ av G allele (homozygoter) (Studie II). Höga sIL6R-nivåer ökade risken för hjärtinfarkt. Sgp130 var en effektmodifierare av sambandet mellan sIL6R och hjärtinfarkt. Det fanns en indikation om en möjlig interaktion mellan höga sIL6R- och låga sgp130- nivåer och risken för hjärtinfarkt (Studie IV). Utifrån ett bredare perspektiv, borde betydelsen av molekylära signalvägar utvärderas i studier med målsättningen att kartlägga komplexa sjukdomar istället för att använda enskilda biomarköranalyser.

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